Studies of gravity-dependent morphogenesis in plants using gravitropic mutants of morning glory

博 士 論 文

Studies of gravity-dependent morphogenesis in plants using gravitropic mutants of morning glory

(アサガオの重力屈性突然変異体を用いた植物形態形成の重力依存性に関する研究)

平成 19 年度

東北大学大学院生命科学研究科
生態システム生命科学専攻

北澤 大典

Studies of gravity-dependent morphogenesis in plants using gravitropic mutants of morning glory

Daisuke Kitazawa

January 2008

Laboratory of Space and Adaptation Biology

Department of Environmental Life Sciences

Graduate School of Life Sciences

Tohoku University

2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan

ACKNOWLEDGMENTS

The author wishes to express a deep gratitude to Professor Hideyuki Takahashi, Associate Professor Nobuharu Fujii and Assistant Professor Yutaka Miyazawa of Tohoku University for their warm and tender instruction, continuous encouragement and constant experimental help throughout this study. The author is grateful to Professor Atsushi Higashitani and Associate Professor Tadashi Sato of Tohoku University for their kind instruction and helpful suggestions. The author also wishes to thank Professor Masao Tasaka and Associate Professor Miyo (Terao) Morita of the Nara Institute of Science and Technology and Associate Professor Hidehiro Fukaki of Kobe University for kindly providing me with valuable vectors and *Arabidopsis* mutants, Professor Shigeru Iida and Associate Professor Atsushi Hoshino of the National Institute for Basic Biology for furnishing the expressed sequence tag information of the Japanese morning glory, and Professor Eiji Nitasaka of Kyusyu University for kindly donating *weeping2* seeds. The author thanks Dr. Minobu Shimizu, Dr. Akie Kobayashi, Dr. Yuko Saito, and members of our laboratory of Space and Adaptation Biology for their support and encouragement. Finally, the author wishes to thank his wife and parents for their emotional support during his academic life.

ABBREVIATIONS

Aux/IAA:	AUXIN/INDOLE-3-ACETIC ACID
CAPS:	cleaved amplified polymorphic sequence
cDNA:	complementary DNA
EST:	expressed sequence tags
GRAS:	GAI, RGA, SCR
GUS:	β-glucuronidase
IPT:	isopentenyltransferase
ORF:	open reading frame
PCR:	polymerase chain reaction
Pn:	*Pharbitis nil*
RACE:	rapid amplification of cDNA ends
RT-PCR:	reverse transcription-PCR
SCR:	SCARECROW
SGR:	SHOOT GRAVITROPISM
SHR:	SHORT-ROOT
t-ZR:	*trans*-zeatin riboside
we:	*weeping*
WT:	wild type
ZIG:	ZIGZAG

CONTENTS PAGE

PREFACE

Plants remain in the place that they germinated throughout their lifetime and therefore cannot escape from various environmental stresses to which they are exposed. The sessile nature of terrestrial plants has meant that throughout evolution they have developed many mechanisms to survive in their ever-changing environment. For example, plants display tropisms in response to various environmental stimuli. Charles Darwin and his son Francis described some of these responses to environmental stimuli more than a century ago in their book, "*The Power of Movement in Plants*" (Darwin and Darwin 1881). Darwin noted that plants had an ability to sense their circumstances so as to orient themselves for optimal growth and development. To date, plants have been shown to respond to diverse environmental signals, such as light, touch, moisture gradients, and gravity (Muday 2001, Massa and Gilroy 2003). Over evolutionary time, plants have adapted to their surroundings with a high degree of plasticity, affording them the ability to respond to ever-changing conditions that provide constant stimulation. One of the most important adaptations that plants have evolved is the ability to sense gravitational forces, and adjusting their growth pattern accordingly. This gravity-dependent growth and development in plants is called gravimorphogenesis or gravimorphism (reviewed in Wareing and Nasr 1958, Takahashi 1997, Hoson and Soga 2003). Gravity is always present in a constant direction and magnitude on Earth. Plants might therefore have utilized gravity as the most reliable signal for their survival.

Gravitropism is a typical example of gravimorphogenesis that enables plants to

precisely orient their photosynthetic organs to light and develop a root system for anchoring themselves and absorbing water and nutrients (reviewed in Blancaflor and Masson 2003). Gravitropism has been extensively studied as an important process in plant physiology and therefore we have an in-depth understanding of the mechanisms by which it occurs. Gravity also influences other aspects of growth and development beside gravitropism. For example, plants synthesize tough cell walls to withstand gravitational forces (Hoson *et al*. 1996, Soga *et al*. 2002), and cucurbitaceous plants develop a peg only on the gravistimulated side of the region between the root and the hypocotyl, which functions to pull the seed coat out (Takahashi 1997, Kamada *et al.* 2003). The following two phenomena have also been hypothesized to be gravity-dependent: oscillatory movements (circumnutation or winding), which helps plant organs grow upward towards suitable environmental cues (Darwin and Darwin 1881), and apical dominance, which is a phenomenon whereby the leading shoot dominates the growth of the axillary buds (Cline 1991). Thus, gravity affects diverse plant morphogenesis besides gravitropism. In order to understand the intimate system of morphogenesis in plants, it is important to clarify the molecular mechanisms of gravimorphogenesis. In contrast to gravitropism, only limited information has been obtained on the mechanisms that regulate gravimorphogenesis because it is more difficult study; gravitropism can be easily demonstrated by reorienting plant organs. Recent developments in experimental technologies such as the three-dimensional clinostat that can imitate the microgravity conditions on Earth, and experiments under real microgravity conditions in space have highlighted the existence of various types of

gravimorphogenesis (Takahashi *et al*. 1999). Nevertheless, the relationship between gravity and plant morphogenesis remains unclear.

In order to explore the mechanisms of gravity-related morphogenesis, I have used gravitropic mutants to develop systems for the study of plant gravimorphogenesis. The characterization of gravitropic mutants is an informative approach because plant phenotypes that genetically link graviresponses could be extracted and their relationship to the graviresponse could be directly analyzed. I selected gravitropic mutants of the Japanese morning glory (*Pharbitis nil* or *Ipomoea nil*), *weeping*, as a model plant. As described above, oscillatory movements (circumnutation and winding) and apical dominance are suggested to be gravity-related phenomenon. These processes are important for plant growth as they facilitate the elevation of the growth points towards suitable environmental conditions. Morning glory has been used as an experimental material for the study of apical dominance because of its short-day property that is advantageous for the study of apical dominance. Furthermore, morning glory is an intriguing material because of the winding (twining) property of its stem that is seen in some climbing plant species. The distinctive features of morning glory have allowed us to perform studies that were not possible using conventional model plants.

In this study, I have characterized two distinct agravitropic mutants of morning glory, *weeping* (*we*) and *weeping2* (*we2*) and identified the genes that are responsible for their abnormal phenotypes. Furthermore, I have used the *weeping* mutant of morning glory together with several gravitropic mutants of *Arabidopsis thaliana* to verify the relationships between the graviresponse and shoot circumnutation, the winding

response and apical dominance, and I have also attempted to clarify the mechanisms regulating gravity-dependent morphogenesis in plants.

GENERAL INTRODUCTION

BACKGROUND AND CURRENT KNOWLEDGE ON GRAVIMORPHOGENESIS IN PLANTS

Most plants are sessile organisms that spend their entire life cycle at the site of their seed germination. Roots anchor plants to the ground and take up water and mineral ions needed for plant growth and development. In contrast, shoots grow above the ground and harvest light energy and carbon dioxide for photosynthesis. In fact, both organs are endowed with sophisticated machinery that allows them to sense the direction of gravity and guide their growth orientation at a specific angle from the corresponding vector. If plants are rotated 90° from their original growth orientation, they perceive gravity and reorient their main growth axis so that once again they grow vertically relative to the gravity vector. This process, called gravitropism, has received a great deal of attention since its recognition two centuries ago (Knight 1806). Recent advances in the disciplines of genetics, physiology, cell biology and biochemistry, together with the adoption of *Arabidopsis thaliana* as a model plant, have brought about several breakthroughs in our understanding of gravitropism in both roots and shoots. Gravity also affects plant growth and development in ways other than gravitropism. For example, clinostation and spaceflight experiments have suggested the existence of various types of gravimorphogenesis; a specialized protuberance in cucurbitaceaous seedlings, the nutational movement of growing organs and the release of axillary buds from apical dominance, all appear to be governed by the graviresponse. In contrast to gravitropism,

however, the mechanisms regulating these gravimorphogenesis processes remain obscure. In this introductory chapter of my thesis, I briefly summarize the current knowledge on gravity related growth and development in plants and finally describe the objectives of this study.

1. GRAVITROPISM

(1) Gravisensing

Gravitropism involves several steps that are organized in a specific response pathway. These include the perception of a gravistimulus (reorientation within the gravitational field), the transduction of this mechanical stimulus into a biological signal, transmission from the site of sensing to the site of response, and a curvature-response which allows the organ tip to resume growth at a predefined set angle from the gravity vector (Masson *et al*. 2002). Recent studies have provided molecular evidence supporting a long-surviving hypothesis about the mechanism of gravitropism, the starch-statolith hypothesis. Sedimentation of starch-filled plastids called amyloplasts along the gravity vector within gravisensing cells (statocytes) in the root and the shoot is the most likely trigger of subsequent intracellular signaling (Kiss 2000). In root gravitropism, the primary sites of gravisensing are located in the root cap. The columella cells consist of three or four layers in the root cap containing amyloplasts that sediment in the direction of gravity. Surgical or genetic manipulation to remove the root cap abolishes the gravitropic response of the root, demonstrating that the root cap is responsible for gravity sensing (Blancaflor *et al*. 1998). In shoots, sediment amyloplasts

have been observed in several kinds of tissues, including the coleoptiles and pulvini of monocotyledonous plants, and the bundle sheaths of hypocotyls and inflorescence stems of dicotyledonous plants. Genetic analysis using *Arabidopsis* have clarified the role of the endodermal cells (endodermis) in shoot gravitropism. The *shoot gravitropism* (*sgr*)*1/scarecrow* (*scr*) and *sgr7/short-root* (*shr*) mutants exhibited no gravitropic response in their inflorescence stems and hypocotyls, whereas their roots showed a normal response to gravity (Fukaki *et al*. 1998). These mutants lack an endodermis in both roots and shoots. Hence, this study demonstrated that the endodermis, which contains sedimentable amyloplasts, is the gravisensing tissue in shoots.

How does the sedimentation of amyloplasts lead to a gravitropic curvature? One current hypothesis is that the sedimentation of amyloplasts disrupts the cytoskeleton by breaking through the dense local networks of actin microfibrils linked to the plasma membrane (Blancaflor and Masson 2003). This physical perturbation is proposed to lead to an activation of mechanosensitive ion channels in the plasma membrane (Yoder *et al.* 2001). In fact, root-cap amyloplasts appear to be surrounded by a dense network of microfilaments, and an actin-based cytoskeleton network occupies the center of the columella cells (Masson *et al*. 2002). Treatment of roots with cytochalasins B and D (drugs that disrupt actin microfilaments) altered the distribution of plastids (Yoder *et al.* 2001); however, it is still difficult to reconcile the role of actin microfibrils in the gravitropic response. Maize roots treated with the actin-polymerization inhibitor, latrunculin-B, exhibited an enhanced gravitropism (Yamamoto and Kiss 2002). Indeed, Hou and colleagues showed that latrunculin-B treated *Arabidopsis* seedling roots display

enhanced gravitropism and exhibit a persistent increase in lateral auxin accumulation accompanied by an increased duration of alkalinization upon gravistimulation (Hou *et al*. 2003, Hou *et al*. 2004). Real-time observation of amyloplast movement in the endodermis strongly suggested that the movement of amyloplasts during gravisensing is not actin-dependent, while the saltatory movement of amyloplasts is actin-dependent and is not essential for gravisensing (Saito *et al.* 2005). Thus, although amyloplast sedimentation is obviously affected by the actin-microfibril network, actin microfibrils might not directly facilitate gravisensing.

Tasaka and his colleagues have proposed the involvement of vacuoles in amyloplast movement (Kato *et al.* 2002b). The *Arabidopsis* mutants, *sgr2*, *sgr3*, *sgr4/zigzag* (*zig*) and *sgr8*/*gravitropism defective* (*grv*)*2*, showing impaired shoot gravitropism have been characterized. The *SGR2* gene was shown to encode a phosphatidic acid-preferring phospholipase A1 that localized to the vacuole and small organelles (Kato *et al.* 2002a). *SGR3* encodes a syntaxin-like protein (AtVAM3) that is localized to the prevacuolar and vacuolar compartments (Yano *et al*. 2003), whereas *SGR4/ZIG* encodes a v-SNARE-like protein (AtVTI11) (Kato *et al*. 2002a). The *SGR8/GRV2* gene encodes a homolog of a DnaJ domain containing protein, which is required for endocytosis (Silady *et al*. 2004). The structure of the vacuoles was altered in these mutants, suggesting a role for these proteins in vacuolar biogenesis and/or function. Furthermore, although amyloplasts were surrounded by flexible vacuolar membranes in wild-type statocytes, they were located in the cytoplasm and did not sediment in mutant statocytes (Kato *et al*. 2002a, Yano *et al*. 2003). Saito *et al.* (2005)

proposed two hypotheses. First, transvacuolar strands may facilitate the smooth translocation of amyloplasts. Second, the vacuolar membrane itself may receive the signal directly from the gravity-oriented movement of amyloplasts; for example, through a change of tension in the membrane as a result of gliding amyloplasts. Taken together, the possible gravisensing system in plants is drawn as follows: in statocytes, when the starch-storage amyloplasts that are surrounded by flexible vacuolar strands sediment due to gravity, the sedimenting amyloplasts disrupt the cytoskeleton linked to the plasma membrane or amyloplasts glide to the surface of the vacuolar membrane. These physical perturbations are proposed to lead to an activation of mechanosensitive ion channels in the plasma membrane and/or vacuolar membrane, and thereby gravitropic second messengers are released.

(2) Signaling systems in gravitropism

Three substances represent the most likely gravitropic second messengers; namely calcium ions, inositol-1,4,5-triphosphate (IP_3) and protons (Blancaflor and Masson 2003). Plieth and Trewavas (2002) reported that after gravitropic stimulation seedlings exhibit an intense period of luminescence of the Ca^{2+} reporter aequorin followed by a steady drop off. Interestingly, other mechanical stimulations don't have the same effect on cytoplasmic [Ca^{2+}] spiking (Plieth and Trewavas 2002). The recently identified *MCA1* gene in *Arabidopsis* is a possible candidate for the mechanosensitive Ca^{2+} channel (Nakagawa *et al.* 2007), which may control cytoplasmic Ca^{2+} influx/efflux in response to gravistimulation. On the other hand, there is an opposing report that steady

state changes in cytosolic Ca^{2+} are unlikely to mediate gravity signaling events in roots. Legué *et al*. (1997) imaged cytosolic Ca^{2+} in root statocytes and although clear touch-related Ca^{2+} transients were observed, these researchers failed to detect any Ca^{2+} changes upon reorientation. They pointed out that touch signaling is well characterized as eliciting Ca^{2+}-dependent events in plants and it is often extremely difficult to divorce the influence of touch responses in gravistimulation protocols. In addition, touch appears to modify the graviresponse (Massa and Gilroy 2003), further complicating the design of experiments to test events that are specifically associated with gravistimulation. To date, direct evidence for a Ca^{2+} change elicited in gravisensing cells in response to gravistimulation remains equivocal. IP_3 is another potential second messenger. When the cereal pulvuni of oat and maize was used as a system to study gravitropically stimulated ion fluctuation, IP_3 levels were shown to increase as much as five fold within ten seconds of gravity stimulation (Perera *et al.* 2006). IP_3 is known as a Ca^{2+} mobilizing second messenger. To date, whether this IP_3 based system is linked to gravisignaling-related Ca^{2+} changes remains undefined.

Changes in pH due to fluxes in protons (H^+) have also been implicated as a signaling mechanism in gravitropism. An alkalization of the cytoplasm of columella cells has been shown to occur within minutes of gravistimulation (Scott and Allen 1999). This is concomitant with an increase in the acidity of the columella apoplast (Fasano *et al.* 2001). These pH changes are absent in mutants that fail to develop amyloplasts or are less sensitive to gravity (Fasano *et al.* 2001). A change in pH could depend upon the changing auxin environment as the rates of pH change seem to follow the rate of auxin

transport (Monschausen and Sievers 2002). Thus, changes in pH as well as in auxin transport occur in the root columella, perhaps triggering a feedback mechanism that influences the activity and distribution of auxin transporters allowing for signaling amplification. Although gravisignaling events mediated by cytosolic Ca^{2+}, IP_3 and pH changes are complicated at present, how gravistimulation facilitates the integration of these signals and how these signaling components are transmitted in order to induce a graviresponse remains to be clarified.

(3) Role of auxin transport in gravitropism

It appears from the aforementioned studies that signaling is ultimately coupled to the distribution of auxin, a phytohormone that is crucial for diverse aspects of plant growth and development. Based on indirect evidence using auxin-inducible promoter elements, several researchers have shown that there seems to be a lateral flow of auxin that is manifested upon gravistimulation (Boonsirichai *et al.* 2003, Ottenschlager *et al.* 2003). This lateral flow would thus lead to a differential growth response, resulting in a gravitropic curvature; this scenario is known as the Cholodny-Went hypothesis. In roots, following gravistimulation, more auxin is directed into the elongation zone at the lower side of the root when compared to the upper side. The resulting auxin gradient causes inhibition of growth on the lower side of the root, and the root thus bends downward. In shoots, gravistimulation also induces auxin accumulation at the lower side compared with the upper side, but the resulting auxin gradient causes promotion of growth on the lower side of the shoot, and the shoots thus bend upward. Directional transport of auxin

has been believed to be a key mechanism that causes such lateral auxin asymmetry (Abas *et al.* 2006). The polar transport system requires transmembrane transporters that can either function to take in auxin from the apoplast (influx facilitator) or can serve to shuttle auxin out of a cell (efflux facilitator). The identified influx facilitators belong to the AUXIN INFLUX CARRIER PROTEIN (AUX)/LIKE-AUX1 (LAX) family of proteins that are related to amino acid transporters (Swarup *et al.* 2004), while components of the efflux facilitator system belong to the PIN-FORMED (PIN)/AGRAVITROPIC (AGR) family and ABC-binding cassette (ABC) transporter family (Noh *et al.* 2003). Many members of the PIN family have been implicated in the gravitropic response of roots and shoots (reviewed in Palme *et al*. 2006), as has AUX1 from the AUX/LAX family of auxin influx facilitators (Marchant *et al.* 1999).

Following gravistimulation, proteasome-dependent degradation of PIN2 at the upper side of the horizontally placed root results in asymmetric distribution of PIN2, indicating that intracellular trafficking and proteolysis of PIN2 are involved in root gravitropism (Abas *et al*. 2006). Many reports suggested that intracellular trafficking and recycling of cargo proteins including PINs play an important role in the gravitropic response (Paciorek *et al*. 2005). In fact, a specific inhibitor of vesicular transport, brefeldin A (BFA), inhibits not only protein recycling but also the gravitropic response (Shin *et al*. 2005). The intracellular localization of PIN3 appears to depend on the root's orientation relative to the gravity vector. PIN3 has been shown to relocalize from a basal to a lateral position within two minutes of gravistimulation (Friml *et al.* 2002). Another protein that appears to function in the formation of a lateral auxin gradient in response to

gravity is ARTERED RESPONSE TO GRAVITY (ARG)1, a ubiquitously expressed J-domain protein (Boonsirichai *et al.* 2003). The *arg-1* mutants fail to redistribute auxin in the root cap when compared to wild-type plants, and they also do not show the characteristic change in pH that is associated with gravitropic stimulation (Boonsirichai *et al.* 2003). Mutations in *ARG1* and its paralog *ARG1-LIKE* (*ARL*) *2* affect root and hypocotyl gravitropism, suggesting that these genes play a role in both root gravitropism and hypocotyl gravitropism. Additionally, mutation of one of the adenosine kinases, ADK1, impairs the relocalization of PIN3 to the bottom membrane of statocytes upon gravistimulation (Young *et al.* 2006). Consequently, *adk1-1* roots cannot develop the lateral auxin gradient across the cap that is necessary for the curvature response. Interestingly, *adk1-1* does not affect gravity-induced cytoplasmic alkalinization of the root statocytes, suggesting either that ADK1 functions at a step between cytoplasmic alkalinization and PIN3 relocalization in a linear pathway, or that the pH and PIN3-relocalization responses to gravistimulation belong to distinct branches of the pathway (Young *et al*. 2006).

In the shoot gravitropism pathway, one of the candidates involved in signal transduction is SGR5 (Morita *et al*. 2006b). The *sgr5-1* mutant exhibits reduced gravitropism in the inflorescence stem but its root and hypocotyl have normal gravitropism. *SGR5* encodes a zinc finger protein with a coiled-coil motif, and is expressed in the endodermis. Interestingly, almost all of the amyloplasts in the *sgr5-1* mutant endodermis sedimented in the direction of gravity. Thus, SGR5 may be involved in shoot gravitropism events such as gravity perception and/or a signaling process

subsequent to amyloplast sedimentation. Another set of potential gravity signal transducers that have been identified in shoots are the *GRAVITY PERSISTENCE SIGNAL* (*GPS*) loci (Nadella *et al*. 2006). These mutants exhibit normal amyloplast sedimentation but have defects in their shoot gravitropic response. The expression of an auxin-inducible reporter gene was redistributed to the lower side of the inflorescence stem in wild type plants after gravistimulation, whereas this did not occur in *gps* mutants, suggesting that GPS functions between gravisensing and lateral auxin transport in response to gravistimulation.

(4) Differential growth in gravitropism

How does redistributed auxin lead to the expansion of only certain plant cells in response to gravitropic stimulation? The answer to this question is probably through the activity of the AUXIN RESPONSE FACTOR (ARF) and AUXIN (Aux)/INDOLE-3-ACETIC ACID (IAA) transcription factor proteins (reviewed in Guilfoyle and Hagen 2007). This notion is consistent with the finding that *non-phototropic response* (*nph*)*4/arf7* mutants and *arf10 arf16* double mutants show a lack of gravitropic response in the stem (Liscum and Briggs 1996). Recent microarray studies corroborate the role of Aux/IAA and ARF activity in the gravitropic response as the expression of *Aux/IAA* and *small auxin up-regulated RNAs* (*SAUR*) family members altered within five minutes of gravity stimulation in the root tip (Kimbrough *et al*. 2004).

In summary, in response to gravity, amyloplast sedimentation within statocytes mechanically stimulates the cytoskeleton and vacuolar or plasma membranes that are

linked to unidentified gravireceptors, which acts as a trigger to commence subsequent steps of the gravitropic response. This physical signal is converted into a biological signal, which determines the localization of auxin transport facilitators, and directs the asymmetric auxin flow. This signaling pathway may involve Ca^{2+} or H^{+} dynamics that lead to changes in auxin flow and establish auxin redistribution. The auxin gradient across the gravistimulated tissues then brings about differential growth bending via auxin-mediated transcription.

Plant organs display not only gravitropism but also other tropisms such as phototropism, hydrotropism, thigmotropism and autotropism (Hoson and Soga 2003); however, on Earth, they are all influenced by gravitropism. For example, interactions between root phototropism and gravitropism have been studied. *Arabidopsis* roots that had been grown in a vertical orientation were illuminated unilaterally so that the light and gravity vectors acted at right angles. In wild-type roots, a new intermediate angle was achieved; however the roots of mutants defective in gravitropism were oriented farther away from the light than the wild type (Okada and Shimura 1994). In addition, the roots of pea and cucumber plants cannot exhibit hydrotropism under 1 *g* conditions, but roots from an agravitropic mutant or those of clinorotated seedlings have enabled hydrotropism to be separated from gravitropism in pea and cucumber plants (Jaffe *et al*. 1985, Mizuno *et al*. 2002). Moreover, plants show spontaneous curvature (autotropism) in microgravity conditions or on a clinostat. For example, rice roots formed a constant mean angle of about 45-50° on a clinostat between the perpendicular base line and the point of radicle emergence (Hoson and Soga 2003). Currently, the mechanisms for

hydrotropism and autotropism are not well understood. It is therefore important to clarify these mechanisms and their interactions with those of gravitropism. This will enhance our understanding of the mechanisms by which plants perceive various environmental stimuli and how they integrate those signals to control their growth orientation.

2. GRAVIMORPHOGENESIS IN CUCURBITACEOUS PLANTS: PEG FORMATION

With the exception of gravitropism, the most drastic and well-studied example of gravimorphogenesis is the gravity-regulated development of the peg in cucurbitaceous seedlings. Cucurbitaceous plants such as the cucumber, melon and gourd develop a specialized protuberance, called a peg, between the hypocotyl and the root (transition zone). The peg plays a role in pulling seedlings out of the seed coat. When cucumber seeds are placed in a horizontal position for germination, the peg develops only on the lower side of the transition zone; however, when cucumber seeds are germinated under microgravity conditions in space, a peg develops on each side of the transition zone (Takahashi *et al*. 2000). Thus, peg formation on the upper side of the transition zone is suppressed in response to gravity when cucumber seedlings are grown in a horizontal position on the ground. Sedimentable amyloplasts are observed in vascular-bundle sheath cells (endodermis) in the transition zone, suggesting that the endodermis contains the gravisensing apparatus for gravity-regulated peg formation. It has been suggested that auxin plays a key role in peg formation (Kamada *et al.* 2000). The endogenous auxin level becomes lower in the peg-suppressed upper side than in the peg-forming lower side of the transition zone (Kamada *et al*. 2003). The application of

exogenous auxin induces additional peg formation on the transition zone even when seedlings are grown in a horizontal position (Kamada *et al.* 2000). Application of the auxin transport inhibitor, 2,3,5-triiodobenzoic acid (TIBA), also induced peg formation on both the upper and lower sides of the transition zone of cucumber seedlings grown in a horizontal position (Kamada *et al*. 2003). This suggests that the horizontal placement of seedlings induces the activation of auxin efflux facilitators on the upper side of the transition zone, which directs auxin flow from the upper side to the lower side, and thereby peg formation on the upper side is suppressed. Application of the anti-auxin, *p*-chlorophenoxyisobutyric acid (PCIB), to cucumber seedlings inhibited peg formation, suggesting that peg formation requires an auxin response/action (Shimizu *et al*. 2007). These results suggest that peg formation on the upper side is negatively controlled by the graviresponse and that auxin plays an important role in gravity-regulated peg formation. Recently, the transcriptional regulation of auxin-responsive genes during gravity-regulated peg formation has been studied. It has been shown that the mRNAs of auxin-inducible genes such as *CsIAA1* and *CS-ACS1* are more abundant in the lower side than in the upper side, whereas the mRNA of the auxin-repressible *CsGRP1* gene is more abundant in the upper side than in the lower side of the transition zone when cucumber seedlings were grown in a horizontal position (Saito *et al.* 2004, Shimizu *et al*. 2006). The auxin response factor CsARF2 may facilitate for the initiation and consequent development of peg by interacting with CsIAA2 (Saito *et al.* 2004). Thus, when cucumber seeds are placed horizontally, auxin is differentially distributed by the action of an auxin efflux facilitator in the transition zone. The endogenous auxin level

subsequently becomes lower in the upper side than in the lower side of the transition zone, which induces various transcriptional systems on both upper and lower sides, and thereby a peg develops only on the gravistimulated lower side.

3. CIRCUMNUTATION AS AFFECTED BY THE GRAVIRESPONSE

Plant organs display oscillatory movements termed circumnutation (reviewed in Johnsson 1979, 1997). This movement is thought to help plant organs grow upwards towards suitable environmental cues. The amplitude, period, and shape of the circumnutation depend on the plant species, the plant organs involved, and the developmental stage of growth. Charles Darwin and his son Francis first described circumnutation in plants in their book "*The Power of Movement in Plants*" (Darwin and Darwin 1881). They performed a comprehensive and comparative physiological survey of circumnutational behavior in over 100 higher plant species and emphatically proclaimed its universality. Circumnutation is functionally linked to other types of movement, such as tropisms and nastic movements (Darwin and Darwin, 1881, Israelsson and Johnsson 1967). Generally, more substantial circumnutation can be observed in the vines and shoots of climbing plants (e.g., morning glory), which need to be anchored for support, compared to the shoots of non-climbing plants (Johnsson and Heathcote 1973, Johnsson 1977). In some plant species, such as morning glory, the stems wind along a support as the plant grows upwards (Darwin 1876, Darwin and Darwin 1881). It is believed that circumnutation provides the motive power for the winding response of climbing plants, and is functionally related to these movements.

Although circumnutation is a biologically intriguing phenomenon, its molecular mechanism still remains obscure.

The most elementary concept, which was essentially in agreement with Darwin, was to define circumnutation as an internally driven process. In contrast, Israelsson and Johnsson (1967) proposed a biophysical theory that could account for nutational oscillations by a gravitropic-overshooting process. The latter hypothesis was tested by examining plant movement in microgravity during a spaceflight experiment. It was shown that sunflower hypocotyls still undergo circumnutation in the gravity-free conditions of space (Brown and Chapman 1984, Brown *et al*. 1990). However, it was noted that the sunflower hypocotyls had a smaller period and amplitude of circumnutation in space than on the ground, indicating that the magnitude of circumnutation does vary depending on the gravitational force (Brown *et al*. 1990). Thus, while gravity may not be required to initiate circumnutation, it may promote the degree of circumnutation.

This conclusion led Johnsson *et al.* (1999) to combine facets of the two original hypotheses into the two-oscillator model. In this model, the internal oscillator causes plant organs to oscillate in the absence of gravity, but the activity of the circumnutation is amplified by a gravitropic feedback system (Johnsson *et al*. 1999). The internal oscillator has been modeled as a growth wave traveling around the elongating organs that could be coupled with the oscillation of growth substances (Johnsson *et al*. 1999). Candidates for the internal oscillator include inorganic ions such as calcium and potassium, phytohormones such as auxin, ethylene, and jasmonic acid, and the circadian rhythm (Johnsson 1997). Decapitation of the shoot apex (auxin source) on pea plants abrogated

shoot circumnutation, and thereafter auxin that was exogenously applied to the cut stump restored it (Tepper and Yang 1996). Treatment of morning glory plants with auxin polar transport inhibitor, *N*-(1-naphthyl) phthalamic acid (NPA), arrested circumnutation (Hatakeda *et al*. 2003), exposure to a high concentration of ethylene gas inhibited circumnutation in the sunflower (Johnsson 1997). Treatment with jasmonic acid also suppressed circumnutation in *Arabidopsis* hypocotyls but not in sunflowers (Johnsson 1997). Thus, plant hormones may play an important role in circumnutation as internal oscillators. Circadian rhythms also influence nutational movement. For example, the *Arabidopsis* mutant *toc1* and *elf3* exhibit defective circadian rhythm and also have an abnormal period of circumnutation (Niinuma *et al*. 2005).

Recent studies have advocated the relationship between circumnutation and an external oscillator other than the gravitropic response. For example, temperature, mechanical stimuli, magnetic force and light are all able to affect circumnutational movement. When light-grown *Arabidopsis* plants were transferred into dark conditions, the curvature angle of circumnutating inflorescence significantly increased (Someya *et al*. 2005). Yoshihara and Iino (2005) reported that red light contributes to circumnutation in rice coleoptiles. In these situations, recent molecular genetic approaches have supported the importance of the graviresponse as the external oscillator during circumnutation. Hatakeda *et al*. (2003) showed that several gravitropic mutants of *Arabidopsis* and morning glory display aberrant circumnutation of shoots. Indeed, Yoshihara and Iino (2006) also reported the importance of graviperception in the circumnutation of rice coleoptiles.

Despite these myriad observations, the mechanism for circumnutation is still unclear. Furthermore, there has been no direct evidence of the involvement of the graviresponse as an external oscillator during circumnutation. This is a controversial issue because the hypocotyls of space-flown sunflowers exhibited circumnutation in microgravity, although the period and amplitude of the movements were smaller (Brown and Chapman 1984).

As Darwin noted, circumnutation is a universal feature of growing plants and is important for plant growth. In order to answer the above questions, further intensive studies are necessary.

4. APICAL DOMINANCE AS AFFECTED BY THE GRAVIRESPONSE

Apical dominance is the central regulatory system for maintaining plant shoot architecture, wherein the growing apical shoot suppresses the growth of axillary buds on the axils of the leaves below it (reviewed in Cline 1991). The axillary meristems have the same developmental potential as the primary shoot apical meristem, and each can therefore form an entire secondary shoot. However, they frequently form only a few leaves before arresting to form a dormant axillary bud. The bud can be subsequently reactivated, producing a branch. This flexibility in axillary meristem activity makes it possible for substantial variations in shoot system architecture to occur in response to the prevailing environmental conditions. Apical dominance is often demonstrated via the removal of the shoot tip, also known as decapitation, which releases axillary buds from apical dominance and initiates their outgrowth. Although the concept of apical

dominance has been known for many centuries, the mechanisms underlying this phenomenon are not well understood and have been the center of debate for over 100 years.

The plant hormones auxin and cytokinin are thought to have major roles in controlling apical dominance, with axillary bud growth being inhibited by the basipetal translocation of auxin but promoted by cytokinin (reviewed in Shimizu-Sato and Mori 2001, reviewed in Ongaro and Leyser 2007). Auxin was the first hormone to be linked to the regulation of apical dominance. Skoog and Thimann (1934) showed that auxin applied to the top of a decapitated plant mimics the effect of the removed apex, preventing bud outgrowth. Since Skoog and Thimann, many researchers over the years have studied the mechanisms by which auxin represses bud outgrowth and controls apical dominance. The shoot apex and young expanding leaves abundantly synthesize indole-3-acetic acid (IAA), one of the active auxin in plants (Ljung *et al*. 2001); thus removal of the shoot apex removes a major auxin source. The auxin is transported basipetally down the shoot by polar transport in the vascular parenchyma (Blakeslee *et al*. 2005). Although it is obvious that basipetally transported auxin from the shoot apex inhibits bud outgrowth, the mechanism of its action in this process is still unclear. It is known that radiolabelled auxin that is applied apically does not enter the bud in any quantity (Booker *et al*. 2003), and in fact the levels of auxin in buds frequently increase when the buds start to be activated (Gocal *et al*. 1991). Furthermore, the direct application of auxin onto the bud does not inhibit outgrowth (Brown *et al*. 1979). Thus, the basipetally moving auxin in the stem must play an indirect role in the inhibition of bud

growth.

Candidates for this auxin-regulated signal include cytokinin. Decapitation leads to increased levels of endogenous cytokinin in the stem and/or xylem sap (Li *et al*. 1995) and increases delivery of cytokinins to the axillary buds (Tanaka *et al*. 2006). Direct application of cytokinin to the axillary buds of intact plants is sufficient to promote their outgrowth (Pillay and Railton 1983). The petunia mutant *sho* (Zubko *et al*. 2002), which was identified by activation tagging, and the *Arabidopsis* mutant *hoc* (Catterou *et al*. 2002) exhibit increased levels of cytokinin and reduced apical dominance. Transgenic plants with elevated cytokinin levels due to the overexpression of the cytokinin biosynthetic gene also exhibit a reduced apical dominance (Medford *et al*. 1989). These observations suggest that there is a preferential role for cytokinin in apical dominance.

The mechanism of action of auxin is indirect and therefore contrasts with the direct effects of cytokinin on bud growth. Interestingly, there is good evidence to suggest that auxin can regulate cytokinin biosynthesis (Nordström *et al*. 2004). This effect of auxin appears to be mediated by a well-characterized auxin signal transduction pathway involving *AXR1*, *TIR1*, and the other *AFB* genes (Leyser 2006). One example of this is that the effect of exogenous auxin on cytokinin synthesis is reduced in loss-of-function mutants of the *AXR1* gene (Nordström *et al*. 2004). This auxin signalling pathway is also required for inhibition of branching. The same *axr1* mutants have increased branching, and their axillary buds are resistant to the inhibitory effects of apical auxin (Stirnberg *et al*. 1999). Tanaka *et al*. (2006) investigated cytokinin content and the expression of the *isopentenyltransferase* (*PsIPT1* and *PsIPT2*) cytokinin biosynthetic genes after

decapitation in the pea. These authors reported that decapitation led to increased cytokinin levels in axillary buds following an increase in *PsIPT* gene expression in the stem, but prior to the increased *PsIPT* gene expression in the bud. Accordingly, cytokinins that have been biosynthesized in the stem may be transported into the axillary buds after decapitation. The increase in cytokinin content and *PsIPT* expression in the stem following this removal of an endogenous auxin is prevented by the application of exogenous auxin (Tanaka *et al*. 2006). Taken together these data suggest that one mechanism for auxin-mediated bud inhibition is through down-regulation of cytokinin synthesis, limiting cytokinin supply to the bud and reducing bud outgrowth.

Apical dominance in plants, although regulated by intrinsic signals such as the developmental stage of the plant, is plastic in its response to various environmental cues such as light and nutrient conditions (Snowden and Napoli 2003), and may also be affected by the influence of gravity. For example, horizontal placement of Japanese morning glories results in the outgrowth of several axillary buds in random locations on the main shoot, while inversion of these plants results in elongation of the buds near the shoot base (Prasad and Cline 1985). Furthermore, bending the upper part of the main shoot releases the axillary bud on the bending-region from apical dominance and initiates elongation of the bud. This release of axillary buds from apical dominance is prevented by clinorotation of the bent plants (Prasad and Cline 1987). These results suggest that the release from apical dominance in morning glory is due, at least in part, to gravistimulation. Similar phenomena have also been observed in agriculture and horticulture. For instance, it is known that shoot bending induces lateral branching and

flower bud development in Japanese pear, cherry and plum trees (Wareing and Nasr 1958, Ito *et al*. 2001). Despite these myriad observations, there has been no direct evidence for a relationship between graviresponse and apical dominance.

It has been proposed that auxin may mediate the influence of gravity on apical dominance. One proposed mechanism by which this occurs is that gravity affects auxin transport and distribution (Wright *et al*. 1978, Wright 1981). However, no change in auxin distribution was observed during shoot bending when the dynamics of endogenous auxin were examined by mass spectrometry (Prasad *et al*. 1993). In addition, there have been no reports delineating a role for cytokinin in axillary bud growth induced by shoot bending. It is therefore unclear whether shoot bending-induced release from apical dominance is governed by the same mechanisms as decapitation-induced release.

Apical dominance is a central concept determining plant shoot architecture. If the graviresponse actually participates in the regulation of apical dominance, it could be surmised that gravity plays an intimate role in establishing growth patterns in higher plants. It is therefore important to investigate gravity-regulated apical dominance and to explore its regulatory mechanisms.

5. OTHER GRAVITY-RELATED MORPHOGENESIS

When rice or barley plants were vertically inverted, elongation of the shoot, leaf blade and leaf sheath was significantly inhibited. In contrast, elongation in the agravitropic-*lazy* rice mutant and the *serpentina* barley mutant was not inhibited by vertical inversion and actually a slight increase in elongation of these organs was

observed (Abe *et al*. 1998). These results suggest that shoot elongation is negatively regulated under 1 *g* conditions on Earth. Wareing and co-workers studied the effect of gravity on trees such as cherry, plum and blackcurrant. In all of the species studied the total annual extension growth of horizontally grown trees was markedly less than that of vertical trees; this effect was accentuated when the plants were rotated. The reduced total growth was due both to a smaller number of internodes and also to reduced internode length (Wareing and Nasr 1958). The mechanisms by which gravity affects the extension growth of plants are not understood well. However, Hoson and his colleagues have shown that the synthesis and metabolism of the cell wall and/or cortical microtubules is probably responsible for gravity-regulated extension growth. Under hyper gravity conditions, plants generally develop a short and thick body. In contrast, under microgravity conditions in space, plants generally developed long, thin bodies (Hoson and Soga 2003). Thus, cell wall rigidity is involved in this response so that plants can resist gravity in the terrestrial environment. The decrease in cell wall extensibility (the increase in cell wall rigidity) in response to hypergravity has also been reported in many plant species. The thickness of cell walls and the molecular mass of matrix polysaccharides underlie the regulation of the rigidity of the cell wall. In *Arabidopsis* hypocotyls, hypergravity increased the molecular mass of xyloglucans, whereas in maize coleoptiles and mesocotyls, hypergravity increased the molecular mass of (1-3)(1-4)-β-glucans (Soga *et al*. 2001). Modification of the metabolic turnover of xyloglucans and (1-3)(1-4)-β-glucans as well as thickening of the cell wall under hypergravity conditions seems to be involved in making the cell wall mechanically rigid to

resist hypergravity. In contrast, space-grown rice coleoptiles and *Arabidopsis* hypocotyls had lower levels of cell wall polysaccharides per unit length than the controls, indicating that microgravity decreased cell wall thickness (Soga *et al*. 2002). The space-grown rice coleoptiles and *Arabidopsis* hypocotyls also exhibited a lower hemicellulosic polysaccharide molecular mass, which was mainly due to a decrease in the molecular size of (1-3)(1-4)-β-glucans in rice and xyloglucans in *Arabidopsis*. In *Arabidopsis* hypocotyls, the activity of xyloglucan-degrading enzymes was increased in space, suggesting that microgravity reduces the molecular mass of xyloglucans by increasing xyloglucan-degrading activity (Hoson *et al*. 2002). Cortical microtubules are also involved in the gravity-resistance response. Soga *et al*. (2006) reported that the hypergravity-induced development of a short and thick body is mediated by reorientation of cortical microtubules from a transverse to a longitudinal direction in azuki bean hypocotyls. In rice coleoptiles that had been grown on a clinostat, the microtubules in epidermal and outer parenchymatous cells on the convex side oriented more transversely than those on the concave side (Hoson *et al*. 2001). The gravisensing and early signal transmission processes in gravity resistance appear to be distinct from those in other forms of gravimorphogenesis. In gravitropism, gravisensing involves the sedimentation of amyloplasts in statocytes and the perceived signal is transmitted to the elongation zones. In gravity resistance, the gravity signal is perceived by mechanoreceptors located on the plasma membrane and the perceived signal triggers a signal transduction pathway within the same cells (Hoson and Soga 2003). The presence of two distinct systems is compatible with the idea that plants have evolved

multiple systems for gravisensing, depending on their evolutionary status.

Another example of this comes from a report that the size and mechanical properties of mature Aceraceae leaves varies in response to gravity (Niklas 1992). Adamchuk (1998) reported that gravity affects chloroplast organization and native chlorophyll form in the mesophyll cells of *Arabidopsis*, and causes alterations in the composition of the pigment-protein complex. In moss, gravity determines the site of protonemata initiation, not the process of initiation itself (Ripetskyj 1998). In wild-type moss protonemata, the direction of filament emergence appeared to be gravimorphic as more than 66% of the new filaments emerged above the horizontal. In contrast, the direction of filament emergence was random in the gravitropic moss mutant, *wwr-1* (Wagner and Sack 1998).

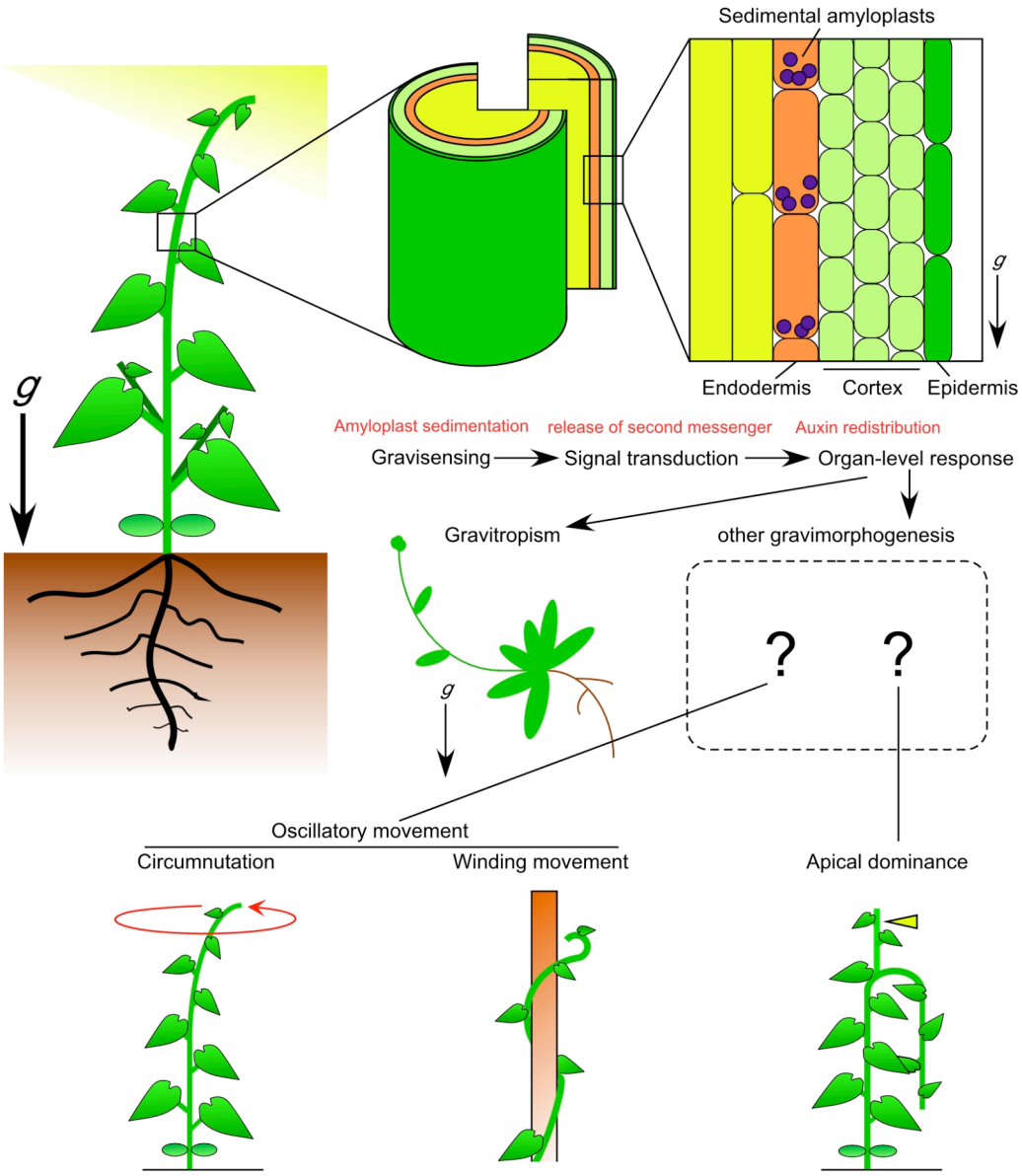
Thus, it has been suggested that gravity affects diverse aspects of plant growth and development; however, the gravity-regulated mechanism controlling these morphogenetic features remains unclear.

OBJECTIVES OF THIS STUDY

As described above, plants have evolved specific morphological features and functions in order to adapt to their terrestrial environment, utilizing gravity as a biological signal. A number of studies have investigated gravitropism in depth, resulting in a greater understanding of the mechanisms by which this phenomenon occurs. Gravisensing cells are likely to be endodermis and columella cells in shoots and roots,

respectively. Numerous studies have suggested that the sedimentation of amyloplasts in gravisensing cells is required for gravisensing, ultimately establishing auxin redistribution that results in bending growth. At present, nevertheless, we do not know how amyloplast sedimentation leads to auxin distribution. Other than the studies on gravitropism, there is very little information available on gravimorphogenesis. Experiments on clinorotation, using gravitropic mutants, and in microgravity suggested possible relationships between gravisensing/graviresponse and various aspects of plant growth and development. However, gravity-dependent morphogenesis in plants still needs to be verified, and its mechanism remains to be clarified (outlined in the introductory figure). Conventional model plants such as *Arabidopsis* and rice may not always be suitable materials for the study of this issue. In this study I have used gravitropic mutants of the Japanese morning glory (*Pharbitis nil* or *Ipomoea nil*) for the study of plant gravimorphogenesis. First, I performed a detailed characterization of two distinct agravitropic mutants of morning glory, *weeping* (*we*) and *weeping2* (*we2*). I then identified the respective mutations that were responsible for their aberrant phenotypes (Chapter 1). Second, I used *weeping* morning glory plants together with gravitropic *Arabidopsis* mutants to study the relationships between the graviresponse and oscillatory movements (circumnutation and winding response) and the release of axillary buds from apical dominance (Chapters 2 and 3). Finally, I attempted to discuss possible roles for the *WEEPING* and *WEEPING2* genes in gravimorphogenesis and the intimate factors governing gravimorphogenesis in plants. Morning glory is a suitable material for the study of circumnutation and the winding (twining) of stems because these phenomena are distinct in this plant species.

The morning glory is also characterized as an absolute short-day plant, which is advantageous for the study of apical dominance as it enabled us to carry out experiments in the vegetative stage without the floral transition of meristems. Studies using *weeping* mutants of morning glory will provide us with conclusive evidence of gravity-governing morphogenesis in plants and a clue to understand the molecular mechanisms regulating this sphenomenon.



Introductory figure. Structural overview of gravity-dependent morphogenesis in shoots. The epidermis, cortex, and endodermis are arranged concentrically from the outside to the inside of the stem. In shoots of dicotyledonous plants, sedimentable amyloplasts are observed in the endodermis. The gravitropic response proceeds through four main steps: first, sensing the direction of gravity; second, conversion of the biophysical signal to a biochemical one (signal transduction); third, transmission of the signal to the responding tissues; and fourth, organ bending. Gravity-dependent morphogenesis other than gravitropism in plants still needs to be verified, and its mechanism remains to be clarified.

CHAPTER 1

Characterization of two *weeping* mutants of morning glory and identification of the genes responsible for their aberrant phenotypes

This chapter is written on referring, in part, to following papers:

Kitazawa, D., Hatakeda, Y., Kamada, M., Fujii, N., Miyazawa, Y., Hoshino, A., Iida, S., Fukaki, H., Morita, M.T., Tasaka, M., Suge, H. and Takahashi, H. (2005) Shoot circumnutation and winding movements require gravisensing cells. *Proc. Natl. Acad. Sci. USA* 102: 18742–18747.

Kitazawa, D., Miyazawa, Y., Fujii, N., Nitasaka, E. and Takahashi, H. (2008) Characterization of a novel gravitropic mutant of morning glory, *weeping2*. *Adv. Space Res*. (in press, doi: 10.1016/j.asr.2007.10.029).

ABSTRACT

In higher plants, gravity is a major environmental cue that governs growth orientation, a phenomenon termed gravitropism. It has been suggested that gravity also affects other aspects of morphogenesis, such as circumnutation, winding growth and apical dominance; however, the relationship between gravity and these phenomena has not been directly demonstrated. In order to verify this issue, it is important to analyze the phenotypes of gravitropic mutants as well as the genes that are responsible for these mutations. Here, I used two distinct mutant lines of morning glory; *we* and *we2,* both of which have defects in shoot gravitropism. These mutants lack the endodermal cells that are required for gravisensing in shoots. I found that one amino acid was inserted into the highly conserved GRAS domain in the *we*-type PnSCR. I performed complementation assays by introducing either the mutant or WT *PnSCR* into *Arabidopsis scr* mutants. *PnSCR* from the WT, but not from *we*, rescued the shoot gravitropism defect and endodermal development. I also found that the *we2*-type *PnSHR1* contained a single nucleotide transition that caused an amber (nonsense) mutation in the GRAS domain. It is known that mRNA level of *SCR* is considerably inhibited in *shr Arabidopsis*. In agreement with this observation, *PnSCR* mRNA was significantly decreased in the organs of *we2* plants compared to WT plants. These results indicate that the aberrant phenotypes in *we* and *we2* are due to mutations in *PnSCR* and *PnSHR1*, respectively. These data suggest that *PnSCR* and *PnSHR1* cooperatively play roles in endodermal development in morning glory.

INTRODUCTION

Morning glory is a traditional horticultural plant in Japan. Many spontaneous mutants affecting the color and shape of leaves and flowers have been isolated since the 17th century, with most appearing around the early 19th century. Before World War II, extensive genetic studies on the Japanese morning glory were conducted by Japanese geneticists. Due to their efforts, more than 200 genetic loci have been described to date. In recent years, many Japanese researchers have constructed an expressed sequence tag (EST) database for morning glory. This data resource is accelerating the usability of morning glory as a model plant for molecular genetic analysis. The shoots of a gravitropic mutant of morning glory, “Shidare-asagao” (*weeping*) display agravitropism while the roots are gravitropically normal. Since its discovery in 1953, the morning glory cultivar *weeping* has been commonly cultivated as an ornamental plant in Japan, and horticulturalists have long been interested in the gene responsible for its abnormal phenotype. A novel *weeping* mutant, *weeping2* (*we2*) was discovered by Eiji Nitasaka of Kyusyu University; this mutant was also generated spontaneously in cultivation. In this chapter, I looked for the genes that are responsible for the aberrant phenotypes of two distinct agravitropic mutants of morning glory, *we* and *we2*, in order to understand the molecular mechanisms underlying morphogenesis in the aerial part of plants. In particular I investigated oscillatory movements and apical dominance, and their relationship with the graviresponse.

The movement of starch-filled plastids called amyloplasts along the gravity vector within gravity-sensing cells (statocytes) in the root and shoot is likely to trigger

subsequent intracellular signaling. The primary sites for sensing gravity are located in the root caps. The columella cells form three or four layers in the root cap and contain amyloplasts that can sediment in the direction of gravity. In shoots, sedimenting amyloplasts have been observed in the bundle sheaths of hypocotyls and the inflorescence stems of dicots. Genetic analysis using *Arabidopsis* have clarified the role of the endodermal cells (endodermis) in *sgr1/scr* and *sgr7/shr* mutants that exhibited no gravitropic response in their inflorescence stems and hypocotyls, whereas their roots showed a normal gravitropic response (Fukaki *et al*. 1998). These mutants lack an endodermis in both roots and shoots. This study demonstrated that the endodermis, which contains sedimentable amyloplasts, is the gravity-perceptive tissue in shoots.

Recently, it was found that *we* plants lack the proper endodermis required for gravisensing; this phenotype is very similar to the *Arabidopsis* agravitropic mutant *sgr1/scr* (see Fukaki *et al.* 1998, Hatakeda *et al*. 2003). Thus, it is possible that the agravitropism of *we* plants is due to a mutation in the *SCR* gene in morning glory. In this chapter, I therefore investigated whether morning glory *SCR* (*PnSCR*) is involved in the *we* phenotype. I also characterized a novel *we2* mutant and sought to identify the gene that is responsible for the aberrant phenotypes of *we2*.

MATERIALS AND METHODS

Plant materials and growth conditions

Pharbitis nil or *Ipomoea nil* Choisy cv. Violet was used as the wild-type (WT) strain throughout these studies. Violet seeds were purchased from Marutane Seed Co., Kyoto,

Japan. *weeping* (*we*) mutant seeds were propagated in our laboratory. *weeping2* (*we2*) is a spontaneous mutant of the morning glory line Q413. The phenotype of Q413 is available on-line through the morning glory web site at Kyusyu University (http://mg.biology.kyushu-u.ac.jp/index.html). In all of the experiments, I used *weeping* mutants that had been crossed twice with WT plants to obtain uniform genetic backgrounds. The morning glory seeds were soaked in sulphuric acid for 30–60 min and then washed overnight with running tap water. They were then sown in a plastic pot filled with commercial soil composite (Kureha Chemical Co., Tokyo, Japan), and grown in a greenhouse.

I used two strains of *Arabidopsis thaliana*, the wild-type ecotype Columbia and the agravitropic mutant *sgr1-1*/*scr-3* (kindly donated from Dr. Masao Tasaka, NAIST), for complementation experiments. Seeds of Columbia and its mutants were sterilized with 5% (v/v) sodium hypochlorite for 5 min, washed three times with sterilized water, mixed in 0.1% (w/v) agar solution, and sown on 1% (w/v) agar containing Murashige and Skoog (MS) medium. The medium was then placed at 23 ± 1 °C for three weeks under white fluorescent light (approximately 50 μmol s^{-1} m^{-2}). Three-week-old plants that had just started to bolt were transplanted into plastic pots filled with vermiculite and grown at 23 ± 1 °C under white fluorescent light (approximately 50 μmol s^{-1} m^{-2}).

Genetic analysis

WT plants were crossed with *we2* mutants, and the resulting F_1 plants were self-pollinated to generate F_2 populations. The gravitropic response of hypocotyls was

examined in both F_1 and F_2 populations.

To determine the allelism of *we2* and *we*, *we2* mutant plants were crossed with *we* mutants, and the resulting F_1 progeny were subjected to a shoot gravitropism assay at 1 week of age.

Gravitropic response assays

In order to assay the gravitropic response in the inflorescence stems of transgenic *Arabidopsis*, intact plants or apical stems that were approximately 4 cm in length were excised from the young primary inflorescence before use. The 4-cm-long stem included shoot apices but excluded all lateral organs. The excised inflorescence stems were pre-incubated in a vertical position under white fluorescent lamps (approximately 50 μmol s^{-1} m^{-2}) at 23°C and were then placed in a horizontal position in the dark at 23°C. The curvature of the stem was measured as the angle formed between the growing direction of the apex and the horizontal baseline. At least eight individuals of each genotype were examined. To examine the gravitropic response of intact inflorescence stems, plastic pots containing plants were placed in a horizontal position in the dark at 23°C.

In order to assay the gravitropic response of morning glory hypocotyls, I used 72-h-old etiolated seedlings that were grown in wet rock wool (Nittoboseki Co., Tokyo, Japan) in a plastic container. Seedlings were gravistimulated by rotating the container 90° in darkness at 25°C. The curvature of the hypocotyls was measured at the indicated times as the angle formed between the direction of growth and the horizontal base line.

To assay the gravitropic response of stems, I used 2-week-old plants that were grown in a greenhouse. Gravistimulation was provided by rotating the pots 90° in darkness at 25°C, and curvatures were measured 24 h after the start of gravistimulation.

To assay the gravitropic response in roots, 48-h-old etiolated seedlings were grown in a vertical position on 0.3% (w/v) Gellan Gum (Sigma Chemicals, St. Louis, MO, USA) plates containing half strength Murashige and Skoog salt (Sigma). Seedlings were placed vertically on new plates and gravistimulated by rotating the plates 90° in the darkness at 25°C. The curvature of the roots was measured at the indicated times as the angle formed between the direction of growth and the horizontal base line.

Phototropic response assays

In order to analyze the phototropic response of morning glory hypocotyls, 72-h-old etiolated seedlings were used that had been grown in wet rock wool in a plastic container. Hypocotyls in plastic containers were covered by a black box with an opening on one side, and were then unilaterally illuminated with approximately 50 μmol s^{-1} m^{-2} white light at 25°C. The curvature of the hypocotyls was determined by measuring the angle formed between the direction of growth and the direction of gravity.

Assays for auxin-responsiveness

To assess the responsiveness of morning glory hypocotyls to exogenous auxin, 72-h-old etiolated seedlings grown in wet rock wool in a plastic container were used. Lanolin containing various concentrations of indole-3-acetic acid (IAA, Wako Pure

Chemical Industries Ltd., Osaka, Japan) was applied to one side of decapitated hypocotyls. These seedlings were grown under dark conditions for 3 h at 25°C. The curvature of hypocotyls was determined as the angle formed between the growing direction and the plumb line.

Microscopy of stem and hypocotyl tissues

Stem segments of 16-day-old *we2* plants and hypocotyl segments of 10-day-old seedlings were fixed in a solution of 4% (w/v) *para*-formaldehyde and 0.25% (v/v) glutaraldehyde at 4°C overnight. The vertical orientation of the tissue segments was maintained during fixation for plants that were grown in the upright position. To examine amyloplast movement in response to gravity, plants were placed in the horizontal position for 3 h; this position was maintained during the fixation process. After fixation, the samples were dehydrated by a series of ethyl-alcohol and *tert*-butyl-alcohol solutions and embedded in paraffin (Paraplast plus; McCormick Scientific, St. Louis, MO, USA). Sections (16 μm thick) were prepared using a rotary microtome and were stained with 0.05% toluidine blue O (Chroma-Gesellschaft, Köngen, Germany) and Lugol's solution (Merck, Darmstadt, Germany). Samples were viewed using an Olympus IX71 microscope (Olympus Optical Co. Ltd, Tokyo, Japan).

For histological analysis of transgenic *Arabidopsis* plants, stem segments were cut from primary inflorescence stems that grew upright after bolting. The segments were fixed in 10% (v/v) formaldehyde, 5% (v/v) acetic acid, and 50% (v/v) ethanol in 0.2-mL tubes under a vacuum, with maintenance of the growth orientation of the stems. After

fixation, the samples were dehydrated by a series of ethanol washes and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. Sections (5 μm thick) were prepared using a rotary microtome and were stained with 0.05% toluidine blue O and observed under an Olympus BX50F microscope (Olympus).

Cloning of full-length PnSCR cDNA

Total RNA was extracted from the shoot apices of WT morning glory plants using TRI reagent (Sigma) according to the manufacturer's instructions. A cDNA of WT morning glory *SCR* was amplified by PCR in the following way. The respective sequences of the upstream and downstream degenerate oligonucleotide primers, 5'-TTCCACATTCTTGCTTCT(C/A)G(C/A)CCTGG-3' and 5'-TGTA(C/A)CCGTC(C/G)GAGGGGAACAT(T/G)CC-3', were based on conserved amino acid regions of the SCR proteins from various plants. First-strand cDNA and amplification were performed using a One Step RT-PCR Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Amplified cDNA fragments were then cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. Based on the sequence information for the *PnSCR* cDNA, a full-length cDNA was obtained using a GeneRacer Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The full-length cDNA fragment from wild type and *weeping* morning glory were amplified by PCR and cloned into pQE31 (Qiagen). DNA sequences were determined using an ABI 310 Genetic Analyzer (Applied

Biosystems, Foster City, CA, USA) following standard procedures.

Full-length *PnSCR* genomic DNA fragments from WT and *weeping* morning glory were amplified by PCR and cloned into a binary vector for subsequent plant transformation.

Cloning of full-length PnSHR cDNAs

Total RNA was extracted from the stems of 14-day-old greenhouse-grown WT morning glory plants, using an RNeasy Plant Mini kit (Qiagen), according to the manufacturer's instructions. First-strand cDNA was synthesized using a ReverTra Ace α kit (TOYOBO, Osaka, Japan), according to the manufacturer's instructions. The expressed sequence tag (EST) database of the Japanese morning glory cv. TKS (Morita *et al*. 2006a) contained two separate contigs that were homologous to *SHR*. These sequences were amplified using the following primers: 5'-CAGCTGCTGTGGATGCTGAA-3' (forward) and 5'-GCTTCAGCTTGGAAGCCCTA-3' (reverse) for *PnSHR1*; and 5'-TTCCAAGCACCCATACCCGCCATC-3' (forward) and 5'-GCTCCTTCGAGTCAACCAGGAAGAT-3' (reverse) for *PnSHR2.* Amplified cDNA fragments were then cloned into pGEM-T Easy (Promega). Based on partial sequence information for the *PnSHR* cDNAs, the full-length cDNAs were obtained using a SMART RACE Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. All of the DNA sequences were verified by sequencing using an ABI 310 Genetic Analyzer (Applied Biosystems) and standard sequencing procedures.

F_2 linkage analysis

For F_2 linkage analysis, *we* or *we2* plants were crossed with WT plants, and the resulting F_1 progeny were self-pollinated to generate F_2 population. Linkage between agravitropism and mutations in *PnSCR* was verified using a cleaved amplified polymorphic sequence (CAPS) marker assay. Dark-grown F_2 seedlings were first distinguished by their gravitropic responses. Genomic DNA was then extracted from each plant, and the DNA fragment including the mutated region was amplified by PCR. The amplified PCR fragment was further digested by *Sau*3AI to discriminate between the WT and *weeping PnSCR*. Linkage between agravitropism and *PnSHR1* mutations in the F_2 population was also verified using a CAPS marker assay. Amplified PCR fragments were digested with *Xsp*I to discriminate between WT and mutant *PnSHR1*. I analyzed twenty-five individual F_2 seedlings displaying either the gravitropic or agravitropic phenotype.

Plant transformation

The *PnSCR* genomic DNA fragments from the wild type and *weeping* cultivars were cloned into a pBI101 vector (Jefferson *et al.* 1987) in which the *GUS* gene had been replaced with the *AtSCR* promoter (kindly donated by Dr. Philip N. Benfey, Duke University). The *PnSCR* genomic DNA fragments were inserted into pBI101*::pAtSCR* downstream of the *AtSCR* promoter. These constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into the *sgr1-1/scr-3* mutant using the floral dip method (Clough and Bent 1998). T_1 plants were selected by their

resistance to kanamycin. The presence of the transgene in these plants was tested by PCR.

Quantitative reverse transcription (RT)-PCR analysis

Total RNA was extracted from the shoot apex and stems of 14-day-old morning glory plants that had been grown in a greenhouse, and hypocotyls and roots from 3-day-old etiolated seedlings. First-strand cDNA was synthesized as described above. Real-time RT-PCR was performed using a MyiQ single-color real-time PCR detection system and an iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. The PCR conditions were as follows: 120 seconds (s) at 95°C; 40 cycles of 10 s at 95°C, 30 s at 55°C, 45 s at 72°C followed by melting curve analysis. Calibration was performed using a plasmid-based cDNA template. Target gene expression was normalized to the expression of the mitochondrial F_1F_0 ATP synthase γ-subunit, which is commonly used as an internal standard in morning glory (GenBank accession no. AB194067). Amplification was carried out using the following primers: SHR1 F1 (5'-CAGCTGCTGTGGATGCTGAA-3') and SHR1 R2 (5'-AACCCACCTAGGAGAGAT-3') for *PnSHR1*; SHR2 F1 (5'-TTCCAAGCACCCATACCCGCCATC-3') and SHR2 R1 (5'-GCTCCTTCGAGTCAACCAGGAAGAT-3') for *PnSHR2*; EST F1 (5'-GAGAATCAATCCTGGGTGGG-3') and SCR Rv3 (5'-AACTCCAATTGCACTACCGCT-3') for *PnSCR*; ATPS F (5'-CAGTGGATCCTGATGACATCCTTAAAAATG-3') and ATPS R

(5'-CTTCCTCGAGTTTTATTTCATCACCATCAG-3') for the ATP synthase γ-subunit.

RESULTS

Characterization of the morning glory SCR gene

Figure 1-1 shows the phenotypes of typical wild-type (WT) morning glory, Violet and the gravitropic mutants of morning glory *weeping* (*we*) and *we2*. The WT morning glory displays gravitropism and a winding response (Fig. 1-1A), whereas the two *weeping* mutants do not display either phenomenon (Fig. 1-1B, C). The stems of *we* and *we2* did not wind around supports even though they were artificially twined to them. When WT plants were placed in a horizontal position, their shoots displayed upright growth (Fig. 1-1D, E). In contrast, the shoots of *we* and *we2* mutants failed to exhibit negative gravitropism, even 24 h after re-orientation (Fig. 1-1F-I). It has been previously shown that hypocotyls of *we* displayed no gravitropic response (Hatakeda *et al*. 2003).

Recently, it was demonstrated that *we* lacks the normal endodermal cell layer that is responsible for the gravitropic response and that *we* plants carry a single recessive mutation (Hatakeda *et al.* 2003). As *Arabidopsis SCR* has been shown to be necessary for proper endodermal development, I examined whether the abnormal phenotypes of *we* were due to either changes in gene expression or loss of function of the morning glory homologue of *SCR* (*PnSCR*). I initially used a PCR-based strategy to isolate a cDNA of the *SCR*-homologous gene from the WT morning glory, Violet (see Materials and Methods). Sequence analysis revealed that an open reading frame (ORF) of the *SCR*-homologous gene has a coding capacity for 783 amino acids (Fig. 1-2). The ORF

encodes a protein with 58% sequence identity to the *Arabidopsis thaliana* SCR protein and *Pisum sativum* SCR protein, and 61% sequence identity to the *Zea mays* SCR protein (Fig. 1-2). I therefore designated this *SCR*-homologous gene *PnSCR* (GenBank accession number AB200391). I carried out a phylogenetic analysis of SCR family members from plants (Fig. 1-10). PnSCR and SCR proteins from other plant species (PsSCR, AtSCR, ZmSCR and OsSCR) form a clade, suggesting that the isolated *PnSCR* encodes an ortholog of SCR. The SCR protein is a member of a family of plant specific transcriptional regulators (Pysh *et al.* 1999), called GRAS, based on the locus designations of three genes for *GAI*, *RGA*, and *SCR* (Pysh *et al.* 1999). The characteristic C-terminal sequence motifs of this protein family—a VHIID domain, two-Leu-heptad repeats, and a SAW domain that is characterized by three pairs of absolutely conserved C-terminal residues (R-E, W-G and W-W) (Pysh *et al.* 1999)—were identified in *PnSCR* (Fig. 1-2).

In order to examine the contribution of *weeping*-type PnSCR to the *weeping* phenotypes, I also isolated a cDNA of PnSCR from *weeping*. Sequence analysis of *weeping PnSCR* (*PnSCRm*) revealed that this gene had one amino acid inserted between amino acids 523 and 524 in the deduced peptide sequence; this insertion occurred in the VHIID motif that is highly conserved among the GRAS family proteins (Fig. 1-2 and Fig. 1-3A). The inserted amino acid was predicted to be an isoleucine.

To investigate whether this mutation is linked to the agravitropism of *weeping,* I carried out a linkage analysis of F_2 generations using a CAPS marker so that the three bases insertion made the cleavage site of *Sau*3AI. As shown in Fig. 3B, all of the F_2

generations showing agravitropism were homozygous for the *weeping*-type *PnSCR*.

Complementation tests for PnSCR using the scr mutant of Arabidopsis

To verify that the *PnSCR* mutation causes the loss of PnSCR function, I introduced the mutant-type *PnSCR* (*PnSCRm*) or the wild-type *PnSCR* (*PnSCR*) into the *Arabidopsis sgr1-1/scr-3* mutant to perform complementation assays. The *sgr1-1/scr-3* mutant lacks shoot gravitropism due to abnormal differentiation of its endodermis. I used the *Arabidopsis SCR* promoter (*pAtSCR*) to drive the introduced genes (Fig. 1-4A). It has been shown that the *AtSCR* gene is expressed specifically in the endodermal cell layer of the root, the hypocotyl, and the inflorescence stem (Di Laurenzio *et al.* 1996, Wysocka-Diller *et al.* 2000).

Inflorescence stems of the wild-type plants responded to gravity and began to bend upward within 30 min, and the curvature reached 90° within 90 min (Fig. 1-4B, F). In contrast, both the parental *scr-3* and *scr-3*/Vector (negative controls) did not respond gravitropically even after 24 h (Fig. 1-4C, F). Transformation of *scr-3* plants with *pAtSCR::PnSCR* restored the shoot gravitropism, providing gravitropic kinetics similar to those of the wild type (Fig. 1-4D, F). Thus, the wild-type PnSCR protein fully complemented the *scr-3* phenotypes. In contrast, transformation of *scr-3* plants with *pAtSCR::PnSCRm* did not rescue the shoot gravitropism, and the plants did not respond to gravity even after 24 h (Fig. 1-4E, F). These results strongly suggest that the abnormal gravitropism in *weeping* is attributable to the loss-of-function mutation of PnSCR.

As described above, AtSCR plays an important role in the differentiation of the

endodermis. If PnSCR also acts on endodermal development in morning glory, it would be expected that *pAtSCR::PnSCR* could rescue the endodermal development of *scr* whereas *pAtSCR::PnSCRm* could not. To test this hypothesis, I examined the inflorescence stem tissues of transgenic plants. In the wild-type shoots, one epidermal cell layer, usually three cortex layers, and one endodermal cell layer were arranged in a concentric manner from the outer side of the stem inward to its core. The endodermal cells were almost uniform in size and shape and contained amyloplasts that had sedimented in the direction of gravity (Fig. 1-5A). In the shoots of both *scr-3* and *scr-3*/Vector, the cell layer containing sediment amyloplasts was not found (Fig. 1-5B). Inflorescence stems of *scr-3/pAtSCR::PnSCR* plants also contained one layer of epidermis, usually three layers of cortex, and one layer of endodermis with amyloplast sedimentation (Fig. 1-5C), and thus were very similar to those of the wild type. In contrast, I found no cell layer containing sediment amyloplasts in *scr-3/pAtSCR::PnSCRm* inflorescence stems (Fig. 1-5D). These observations confirm our hypothesis that PnSCR is necessary for the differentiation of endodermis and graviresponses in morning glory.

Phenotypic and genetic characterization of we2 mutants

I next focused on the gene that was responsible for agravitropism in *weeping2*. Gravitropism and winding were normal in WT morning glory (Fig. 1-1A, D, E), but were not evident in *we2* mutants (Fig. 1-1C, H, I). In order to characterize the gravitropic response of *we2* plants in more detail, I carried out a time course analysis of the

gravitropic responses of *we2* hypocotyls and roots. Hypocotyls of WT plants responded to gravity and began to bend upward within 3 h after re-orientation; their curvature reached approximately 90° within 6 h (Fig. 1-6A). In contrast, the hypocotyls of *we2* mutants showed only a slight gravitropic curvature 9 h after re-orientation (Fig. 1-6A). At 24 h after re-orientation, the bending was very slight, due to the weight of the hypocotyls. The gravitropic curvature of *we2* roots was not significantly different from WT roots (Fig. 1-6B). These results indicated that the *we2* mutation affects the gravitropism of stems and hypocotyls, but does not affect the roots of morning glory. Shoots of *we2* mutants were capable of phototropic responses (Fig. 1-6C). In addition, hypocotyls of *we2* mutants showed an almost equal sensitivity to the unilateral application of IAA compared to WT, curving away from the auxin source (Fig. 1-6D). These observations suggested that *we2* is specifically defective in the shoot gravitropic response, rather than in asymmetric growth itself.

To characterize the genotype of the *we2* mutant, I crossed *we2* mutant plants with WT plants and analyzed the gravitropic responses of the resulting progeny. The hypocotyls of all of the F_1 progeny had a normal gravitropic response. In the F_2 generation, the abnormal gravitropic response segregated into approximately a quarter of the progeny (Table 1-2). These results indicated that *we2* plants carry a single recessive mutation. I also performed a complementation test by crossing *we2* mutants to *we* mutant plants. As shown in Table 1-2, all of the F_1 progeny showed a normal gravitropic response, indicating that *we2* and *we* occupy separate genomic loci.

Histological analysis of we2 mutants

During gravitropism, the movement of amyloplasts (starch-filled plastids) within statocytes such as root columella cells and shoot endodermal cells in the direction of the gravitational force is thought to trigger the gravitropic response (Kiss *et al*. 1989, Sack 1997, Morita and Tasaka, 2004). It was previously shown that *we* mutants had abnormal endodermal cells, and that this resulted in agravitropic shoot growth (Hatakeda *et al.* 2003). Because *we2* was not allelic to *we*, I examined the endodermal cells and sedimented amyloplasts in *we2* plants in order to gain insight into the gene responsible for the *we2* phenotype. Histological analysis of the stem and the hypocotyl tissues of WT and *we2* mutants revealed that in WT plants, the vertically oriented stems contained sediment amyloplasts on the floor of the endodermal cells (Fig. 1-7A). In contrast, *we2* mutant stems did not appear to have any sediment-amyoplast-containing endodermal cells with the characteristic shape and location of the endodermal cells of WT plants (Fig. 1-7D). When WT plant stems were placed in a horizontal position, the amyloplasts in the endodermal cells moved to the new “floor” of the cells (Fig. 1-7B, E, G). This amyloplast movement was not observed in the stems of *we2* mutants (Fig. 1-7C, F, J). I also examined hypocotyl sections of WT and *we2* plants that were placed in a horizontal position. Endodermal cells containing sedimented amyloplasts were readily detectable in WT (Fig. 1-7H, K), but not *we2* plants (Fig. 1-7I, L). In contrast to the stems of *we2* plants, the hypocotyls exhibited an abnormal endodermis-like cell layer that lacked sedimented amyloplasts in an outer layer of the vascular tissue. These results suggested that the abnormal gravitropism observed in *we2* mutants is due to a lack of endodermal cells,

and that *WE2* is involved in the development of endodermal cells, similar to *WE*/*PnSCR*.

Characterization of the gene responsible for abnormal we2 mutant phenotypes

In *Arabidopsis*, the cooperative action of *SCARECROW* (*SCR*) and *SHORT-ROOT* (*SHR*) plays a key role in endodermal development in the shoots and roots (Fukaki *et al*. 1998, Helariutta *et al.* 2000). As described above, I showed that the morning glory homolog of *SCR* (*PnSCR*) is responsible for the abnormal phenotype of *we* mutant plants, which lack a proper endodermis. Since *we2* and *we* occupy separate genomic loci, I was interested in whether the abnormal phenotype of *we2* mutants was caused by a mutation of the morning glory homolog of *SHR*. The EST database of the Japanese morning glory contained two sequences that were homologous to *SHR*. I therefore used a PCR-based strategy to isolate the corresponding cDNAs from WT plants (see Materials and Methods). Sequence analysis revealed that the open reading frames (ORFs) of the two putative *SHR*-homologs encode proteins of 412 and 505 amino acids (aa) (Fig. 1-8A). The proteins contained 50% and 66% sequence identity to *Arabidopsis thaliana* SHR proteins, and 44% and 55% sequence identity to *Oriza sativa* SHR1. I designated the two putative morning glory *SHR* homologs *PnSHR1* and *PnSHR2,* respectively (GenBank accession numbers. AB330311 and AB330312, respectively). I then carried out a phylogenetic analysis of the SHR family members in plants (Fig. 1-9). The PnSHRs and SHR proteins from several plant species (*A. thaliana* SHR; AtSHR, *Medicago truncatula* SHR; MtSHR, *O. sativa* SHR; OsSHR1 and OnSHR2) formed a clade, which suggested that *PnSHR1* and *PnSHR2* encode orthologs of SHR.

To determine whether *PnSHR*s were involved in the *we2* phenotype, I isolated full-length cDNAs of *PnSHR1* and *PnSHR2* from *we2* plants. Sequence analysis of *we2* *PnSHR1* and *PnSHR2* revealed that *PnSHR1* contained a single nucleotide transition (T-to-A transition) that converted the leucine 76 codon (Leu 76) into an amber codon (Fig. 1-8, 1-10A). I found no alterations in *PnSHR2* in *we2* plants. The nucleotide transition in *PnSHR1* occurred in the GRAS domain. In order to determine whether the mutation identified was linked to the agravitropism phenotype of *we2* plants, I performed a linkage analysis of F_2 populations using a cleaved amplified polymorphic sequence (CAPS) marker, in which the T-to-A substitution generated an *Xsp*I cleavage site. As shown in Fig. 1-10B, all of the F_2 plants that exhibited agravitropism were homozygous for the *PnSHR1* transition mutation, indicating a strong linkage between this mutation and the agravitropic phenotype of *we2.*

Expression of PnSHRs and PnSCR transcripts

I next examined *PnSHR1* and *PnSHR2* mRNA levels in WT and *we2* plants. Quantitative RT-PCR analysis revealed that the level of *PnSHR1* mRNA was decreased in the organs of *we2* plants. In contrast, the expression of *PnSHR2* was similar to that observed in the WT (Fig. 1-11, upper and middle panels). This result suggested that the premature truncation of *PnSHR1* in *we2* plants contributes to their agravitropic phenotype. SHR directly regulates *SCR* transcription in *Arabidopsis* by binding to the promoter region of *SCR* (Levesque *et al.* 2006). In an *shr* mutant background, the level of transcription of *SCR* is considerably inhibited (Helariutta *et al*. 2000). To determine

whether the expression of *PnSCR* was affected in *we2* mutants, I performed quantitative RT-PCR analysis of *PnSCR* mRNA levels in the organs of *we2*. *PnSCR* mRNA was significantly decreased in the stems and the hypocotyls of *we2* plants compared to WT plants (Fig. 1-11, lower panel), which suggested that in *we2* mutant plants, PnSHR1 is unable to activate the transcription of *PnSCR*. These results suggested that *WE2* corresponds to *PnSHR1*, and is required for the development of endodermal cells.

DISCUSSION

In order to gain a molecular basis for elucidating a detailed mechanism for shoot gravimorphogenesis, I identified the genes responsible for the aberrant phenotypes of two *weeping* mutants. I found that one amino acid was inserted into the highly conserved GRAS domain in *we*-type PnSCR and that *we2*-type *PnSHR1* contained a single nucleotide transition that caused an amber (nonsense) mutation in the GRAS domain; these mutations caused abnormal endodermal differentiation in both *weeping* mutants. These results implied that the aberrant phenotypes in *we* and *we2* plants are due to the mutations in *PnSCR* and *PnSHR1*, respectively (Kitazawa *et al*. 2005, Kitazawa *et al*. 2008).

SHR and SCR are key regulators of shoot and root radial patterning (Di Laurenzio *et al*. 1996, Fukaki *et al*. 1998, Helariutta *et al.* 2000) and stem cell maintenance (Sabatini *et al*. 2003). The *Arabidopsis scr* and *shr* mutants have an abnormally differentiated endodermis and lack the shoot gravitropic response (Fukaki *et al*. 1998). SHR is a transcription factor that is expressed in the stele, and then transported into the

adjacent cell layer where it regulates SCR transcription and endodermal specification (Nakajima *et al*. 2001). SCR is predominantly expressed in the endodermis, the cortex/endodermis initial (CEI) cell, and the quiescent center (QC), and is required for the asymmetric cell division that gives rise to the cortex and endodermis (Di Laurenzio *et al*. 1996, Gallagher *et al*. 2004). Thus, SHR and SCR cooperatively regulate endodermal development. In *scr-3/pAtSCR::PnSCR* plants, endodermal development in inflorescence stems was restored to that of the wild type. The phylogenetic analysis of SCR/SHR family members revealed that PnSCR fell within a clade including SCR proteins from other plant species. These observations confirm that PnSCR is orthologous to SCR and that the abnormal differentiation of the endodermis in *weeping* is due to the mutation in *PnSCR*. Of the four *scr*-mutant alleles of *Arabidopsis* that have been isolated, all of the gene products are truncated by a frameshift mutation or T-DNA insertion (Di Laurenzio *et al*. 1996, Fukaki *et al*. 1998). The in-frame mutation in the VHIID motif of *PnSCR* indicates that the *weeping* mutation is a novel type of mutation among *scr* mutants. I do not know how this three-base-pair insertion occurred in the *PnSCR* at present, but I speculate that it is a footprint generated by an excision of a DNA transposon in the *Tpn1* family belonging to the CACTA superfamily. In fact, the *Tpn1*-related elements are thought to act as major spontaneous mutagens for the generation of various floricultural traits in the Japanese morning glory and are shown to generate three-base-pair insertions (Fukuda *et al*. 2000, Hoshino *et al*. 2001).

I also found that *we2* displays an impaired gravitropic response in the stems but shows a reduced gravitropic response in the hypocotyls, in contrast to that of *we,* which

completely lacks a gravitropic response in the shoots. In contrast, *we2* exhibited a normal gravitropic response in the roots compared to the WT; this was also observed in in *we*. In both gravitropism and phototropism, asymmetric growth is believed to be caused by asymmetric auxin distribution (Liscum and Briggs 1996). If *we2* mutants were not capable of auxin-dependent asymmetric growth, it would indicate that tropic phenomena themselves were defective in these plants. When auxin (IAA) was exogenously applied to seedlings, *we2* mutants showed almost the same auxin sensitivity as wild-type morning glory. Additionally, the phototropism of hypocotyls in *we2* mutants was normal. These results suggest that *we2* is able to regulate shoot elongation in response to endogenous auxin. I also showed that *we2* plants lack normal endodermal cells, and that *PnSHR1* in *we2* mutants has a single nucleotide substitution, which was linked to the agravitropism phenotype of *we2*. Moreover, *PnSCR* expression was remarkably reduced in *we2* mutants, which strongly suggests that *WE2* corresponds to *PnSHR1.* The *PnSHR1* mutation in *we2* plants introduced an amber codon at Leu 76. Thus, the abnormal differentiation of the endodermis observed in *we2* mutants may be due to the expression of a truncated *PnSHR1* gene product. Indeed, root gravitropism, phototropism and auxin sensitivity are normal in *Arabidopsis shr* mutants (Morita *et al*. 2007). The agravitropic phenotype of *we2* mutants was similar to that of the *Arabidopsis* mutant *endodermal-amyloplast less 1* (*eal1*)*,* which is allelic to *SHR* (Fujihira *et al*. 2000, Morita *et al.* 2007). In *eal1* plants, gravitropism is completely absent in inflorescence stems, and is reduced in hypocotyls, whereas the roots show normal gravitropism. In contrast to *we2*, the stems and hypocotyls of *eal1* plants contain an endodermal layer.

However, amyloplast-like plastids that did not sediment in the direction of gravity are observed in *eal1* inflorescence stems. Therefore, the *we2* mutation appears to be more severe than that of *eal1*. *PnSHR1* is one of two morning glory homologs of *SHR.* Thus, endodermal development in morning glory may also involve *PnSHR2*. *SHR* is present as a single copy in the *Arabidopsis* genome, which suggests that there are different mechanisms of endodermal development involving SHR and SCR in *Arabidopsis* and morning glory. The rice genome contains two *SHR* homologs, *OsSHR1* and *OsSHR2* (Fig. 1-8, 1-9). *OsSHR1* is expressed in the root stele, similar to *Arabidopsis SHR* (Cui *et al.* 2007). In contrast, expression of *OsSHR2* is not localized to the stele, but to the endodermis and part of the cortex (Kamiya *et al*. 2003). These observations suggest that OsSHR1 is the functional homolog of AtSHR, and that OsSHR2 has functions other than in endodermal development. In morning glory, I cannot rule out the possibility that PnSHR2 is a functional homolog of AtSHR that is not expressed in the stele. Alternatively, PnSHR1 and PnSHR2 may have redundant functions in endodermal development, through a mechanism that is distinct from that of *Arabidopsis.* These possibilities seem likely based on my observation that there was a higher level of expression of *PnSCR* in the shoot apex and hypocotyls of *we2* plants compared to other organs, which is likely an effect of PnSHR2 (Fig. 1-11). Nevertheless, it is probable that PnSHR1 functions upstream of PnSCR during endodermal development in morning glory.

In this chapter, I succeeded in obtaining two separate loci controlling the differentiation of gravisensing/graviresponsive apparatus in the shoots of morning glory,

a typical climbing plant (Kitazawa *et al*. 2005, Kitazawa *et al*. 2008). This discovery will provide a new insight into the study of the graviresponse by the identification of genes that function downstream of PnSCR and PnSHR1. *weeping* mutants could be a powerful tool to investigate the role of the endodermal function during gravimorphogenesis in the aerial part of the plant.

Table 1-1. List of gene-specific primers used for real-time RT-PCR

Gene	Forward primer sequence	Reverse primer sequence
PnSHR1	5'-CAGCTGCTGTGGATGCTGAA-3'	5'-AACCCACCTAGGAGAGAT-3'
PnSHR2	5'-TTCCAAGCACCCATACCCGCCATC-3'	5'-GCTCCTTCGAGTCAACCAGGAAGAT-3'
PnSCR	5'-GAGAATCAATCCTGGGTGGG-3'	5'-AACTCCAATTGCACTACCGCT-3'
PnATPS	5'-CAGTGGATCCTGATGACATCCTTAA-	5'-CTTCCTCGAGTTTTATTTCATCACC-
	-AAATG-3'	-ATCAG-3'
PnIPT1	5'-TAACAAGGTCACCGACGAGG-3'	5'-AATGGCGTCTTCCAGTAGCC-3'
PnIAA1	5'-AATGACGGCGAAGCGGTGAA-3'	5'-GGATCAGAAGCCATTGGACT-3'
PnPIN1	5'-CCTCTTGTCAACTCCTCAAA-3'	5'-GTCCCTTTCGCCTTTCTCTT-3'
PnPIN2	5'-GAGGCTGAGGTGAAAGAGGA-3'	5'-GAGCCTTGTCATCACACTTG-3'
PnPIN3	5'-CAGAGGCTCAAGCTTTAACC-3'	5'-CATGACACTTGCAGGAGGCA-3'
PnPIN4	5'-AAGAGGCTCTAGCTTCAACC-3'	5'-TTCCTCCACACCATAATCAG-3'

Table 1-2. Genetic analysis of *we2* mutants

Crosses	Generation	Number of plants		$\chi^{2\,a}$
		Normal gravitropism	Abnormal gravitropism	
we2 × Violet	F_1	24	0	
we2 × Violet	F_2	113	37	0.0089
we × *we2*	F_1	36	0	

Gravitropic response of the hypocotyls of the progeny of the crosses listed above. [a] χ^2 was calculated based on an expected ratio of 3:1 normal to abnormal.

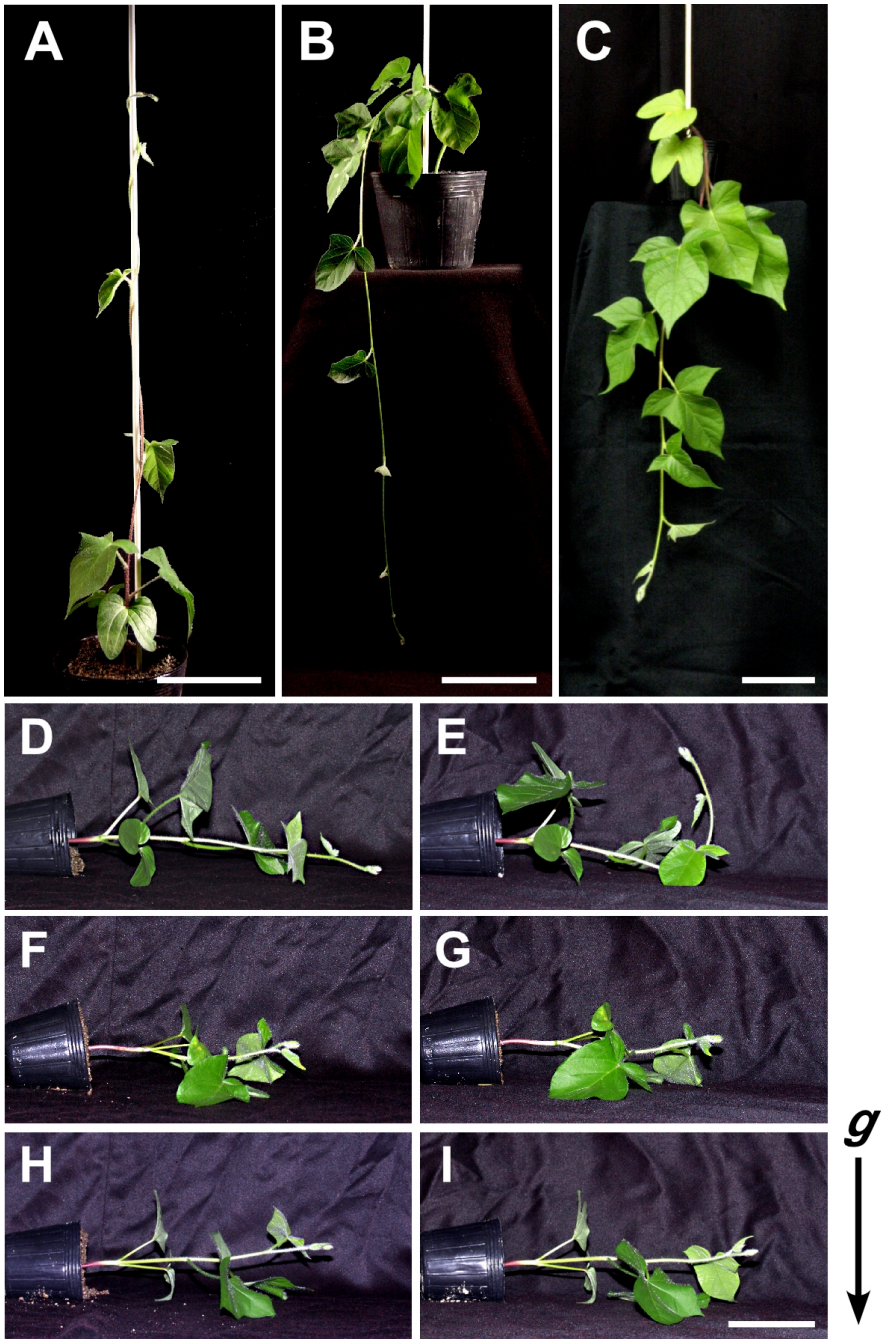


Fig. 1-1. Growth of the Violet morning glory cultivar (WT) and the *we* and *we2* mutants. Representative photographs of two-week-old WT (A), *we* (B) and *we2* (C) plants. The shoots of WT plants that were placed in a horizontal position showed upward bending (D, E), while the shoots of *we* (F, G) and *we2* (H, I) plants placed in a horizontal position failed to bend gravitropically. Photographs were taken 0 h (D, F, H) and 24 h (E, G, I) after re-orientation of the plants. The arrow (*g*) indicates the direction of the gravitational force. Scale bars: 8 cm.

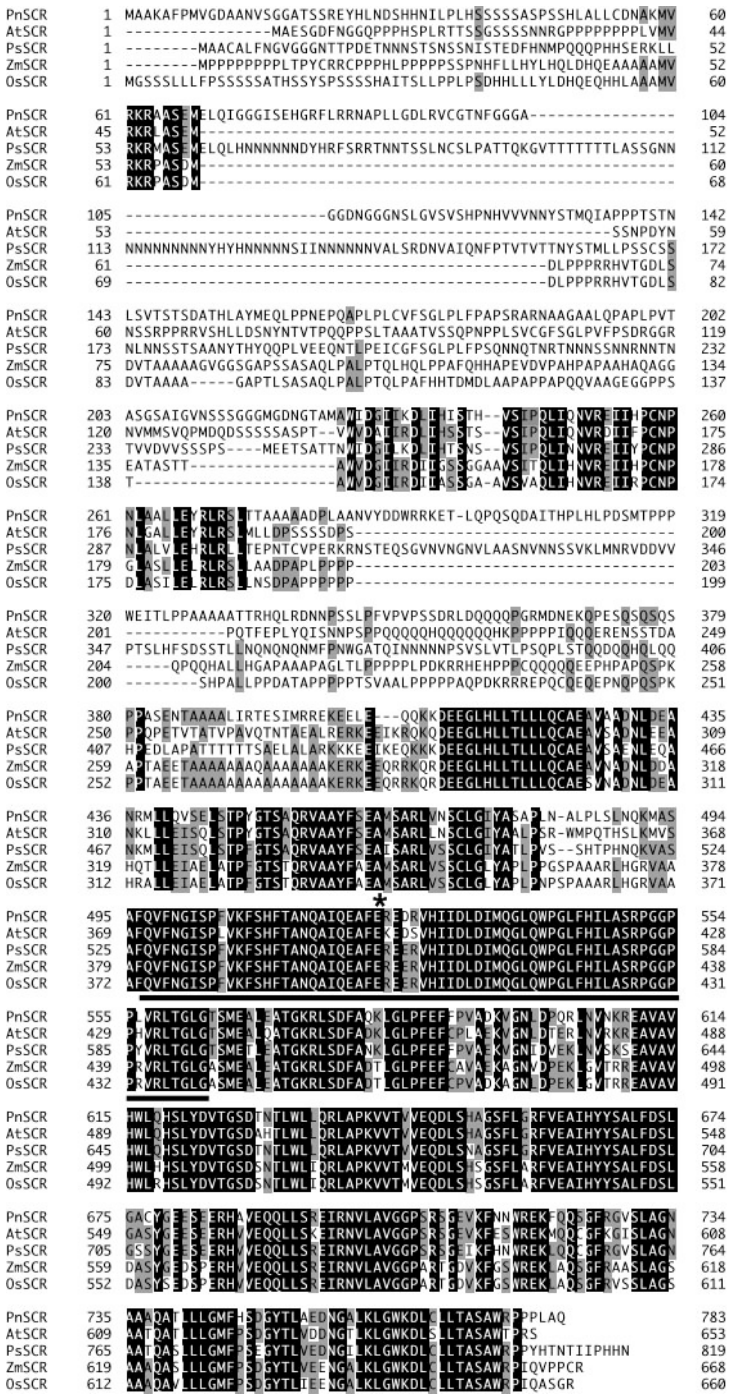


Fig. 1-2. Amino acid sequence alignments of SCR proteins. Alignment of the amino acid sequence of PnSCR and other SCR proteins. Identical and similar amino acids are highlighted in black and gray boxes, respectively. The VHIID motif is underlined. The star symbol indicates the site of the mutation in the *weeping*-type PnSCR. GenBank accession numbers: morning glory SCR (PnSCR), AB200391; *Arabidopsis* SCR (AtSCR), AB010700; pea SCR (PsSCR), AB048714; maize SCR (ZmSCR), AF263457; rice SCR (OsSCR), AB180961.

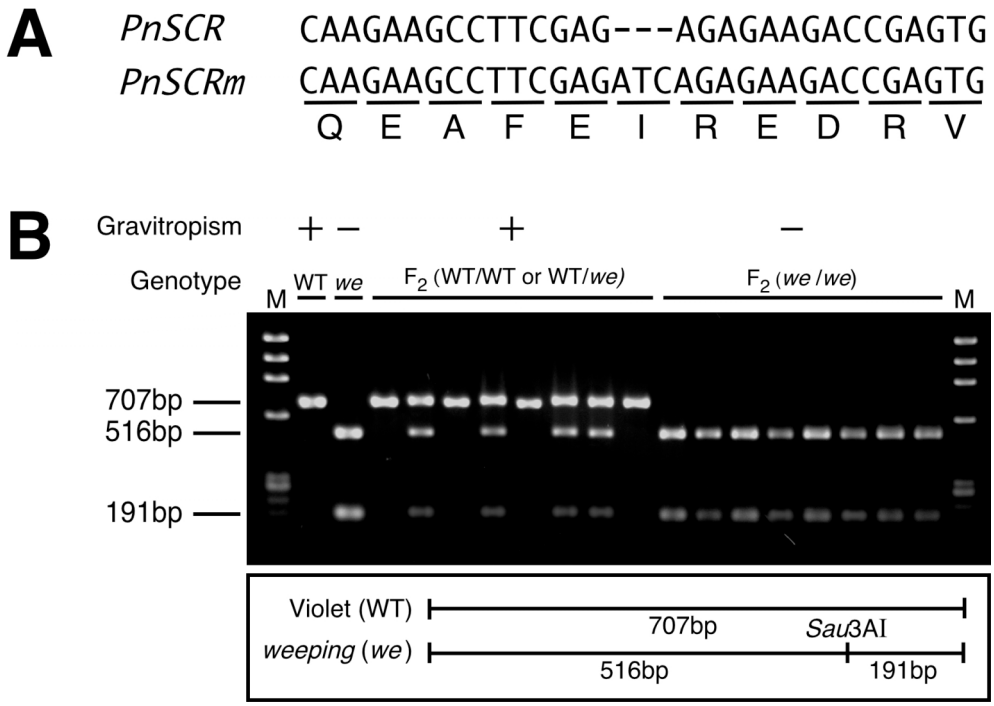


Fig. 1-3. Comparison of the VHIID motifs in Violet-type *PnSCR* and *weeping*-type *PnSCR*, and F_2 linkage analysis between agravitropism and the *PnSCR* mutation. (A) Part of the VHIID motif in Violet-type *PnSCR* (*PnSCR*) and *weeping*-type *PnSCR* (*PnSCRm*). (B) Linkage between agravitropism and the mutation. Dark-grown F_2 seedlings were distinguished by their gravitropic responses. *PnSCR* was amplified by PCR from genomic DNA isolated from each F_2 seedling. The PCR products were digested with *Sau*3AI and analyzed by agarose gel electrophoresis. Abbreviations: M, molecular weight marker (øX174/*Hae*III); WT, Violet; *we*, *weeping*. Data from eight F_2 individuals of each phenotype are shown.

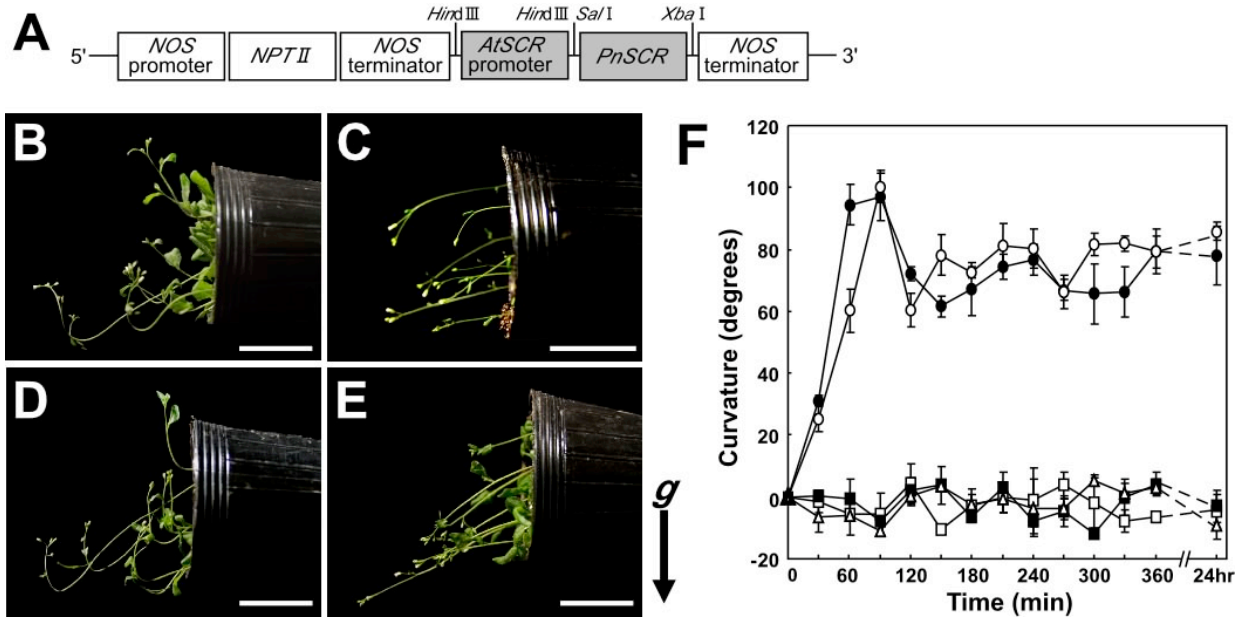


Fig. 1-4. Shoot gravitropism in wild-type (WT), *scr,* and transformant *Arabidopsis*. (A) The pBI101ΔGUS*::pAtSCR::PnSCR* construct that was used for complementation assays. (B) Gravitropism of wild-type *Arabidopsis*. (C) Gravitropism of *sgr1-1/scr-3* with pBI101ΔGUS*::pAtSCR*. (D) Complementation of *sgr1-1/scr-3* gravitropism with *pAtSCR::PnSCR*. (E) Complementation of *pAtSCR::PnSCRm*. WT and transgenic plants were placed in a horizontal position at 23°C for 6 h in the dark. Arrow (*g*) indicates the direction of gravitational force. Bars = 2 cm. (F) Time course of the gravitropic responses of the excised inflorescence stems of WT, *sgr1-1/scr-3,* and transgenic plants: WT (open circles), *scr*/*PnSCR* (closed circles), *scr*/*PnSCRm* (open squares), *scr*/Vector (closed squares), *sgr1-1/scr-3* (open triangles). The excised inflorescence stems were gravistimulated by being placed in a horizontal position at 23°C in the dark. Data represent means ± SE.

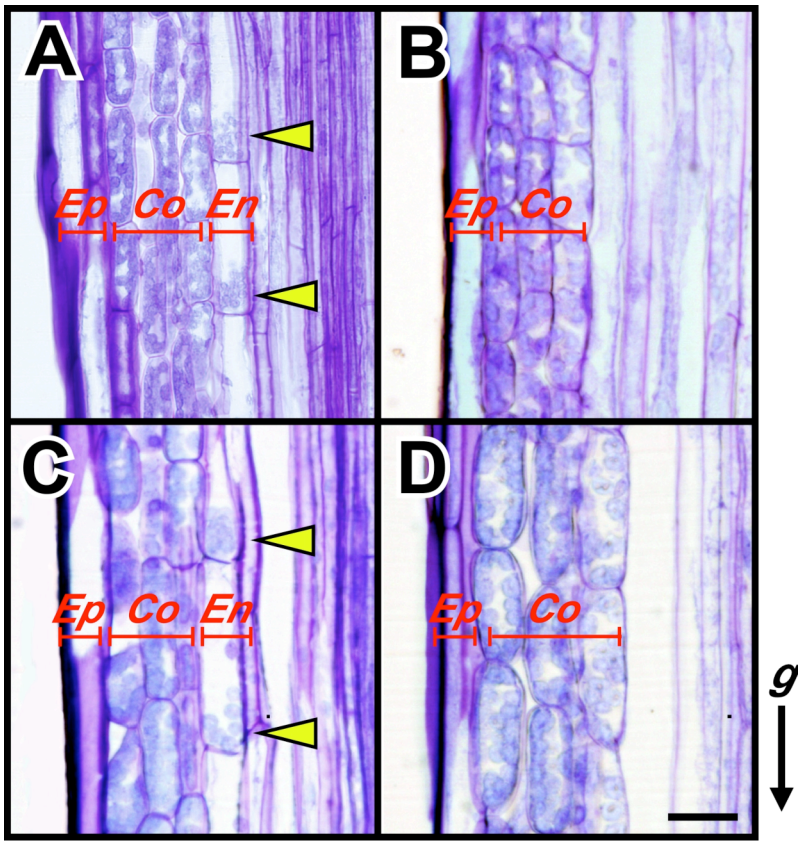


Fig. 1-5. Histological analysis of wild-type (WT) and transformant *Arabidopsis* stained with toluidine blue. Longitudinal sections of inflorescence stems of WT (A), *scr*/Vector (B), *scr*/*pAtSCR::PnSCR* (C), and *scr*/*pAtSCR::PnSCRm* (D) *Arabidopsis*. Arrowheads indicate the sedimented amyloplasts. The arrow (*g*) indicates the direction of gravitational force. Bar = 20 μm. Abbreviations: *Ep*, epidermis; *Co*, cortex; *En*, endodermis.

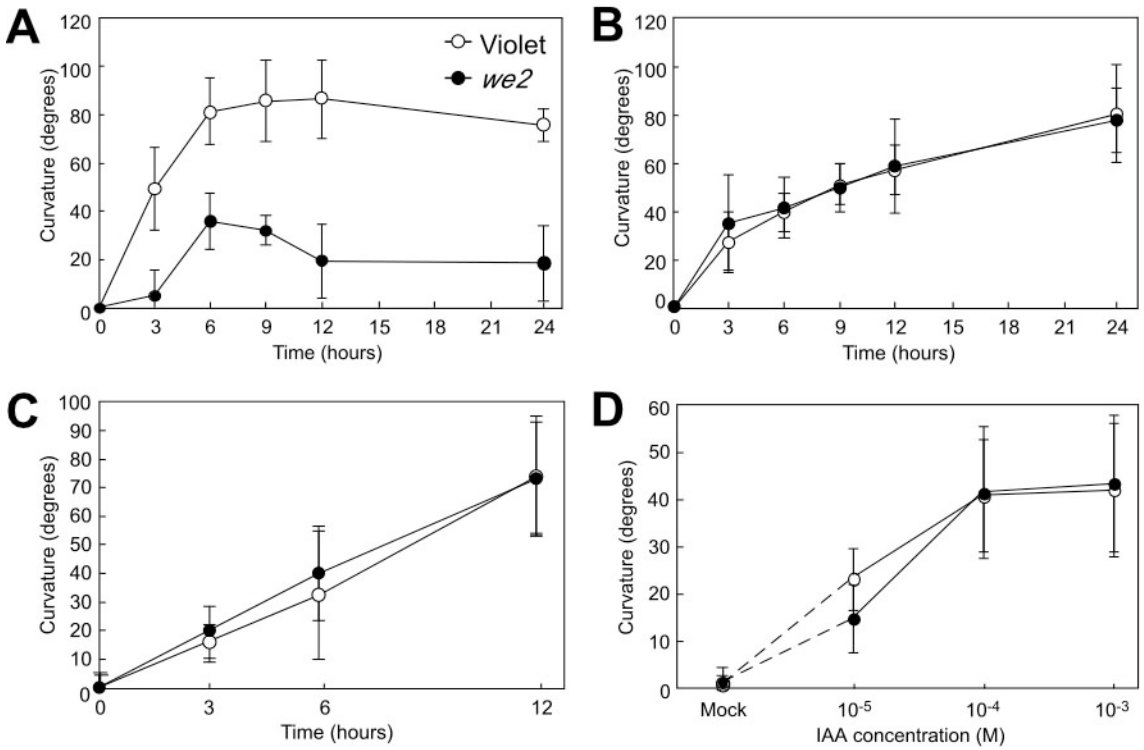


Fig. 1-6. Tropic responses of the hypocotyls and roots from WT and *we2* mutant plants. Time course of the gravitropic response in hypocotyls (A) and roots (B). Time course of phototropic responses (**C**) and dose-response curves from a hypocotyl growth-curvature test using lanolin containing IAA (**D**). Data represent the mean and standard deviation of the curvatures. Twenty-four plants of each genotype were examined. Open and filled circles indicate WT and *we2* mutants, respectively.

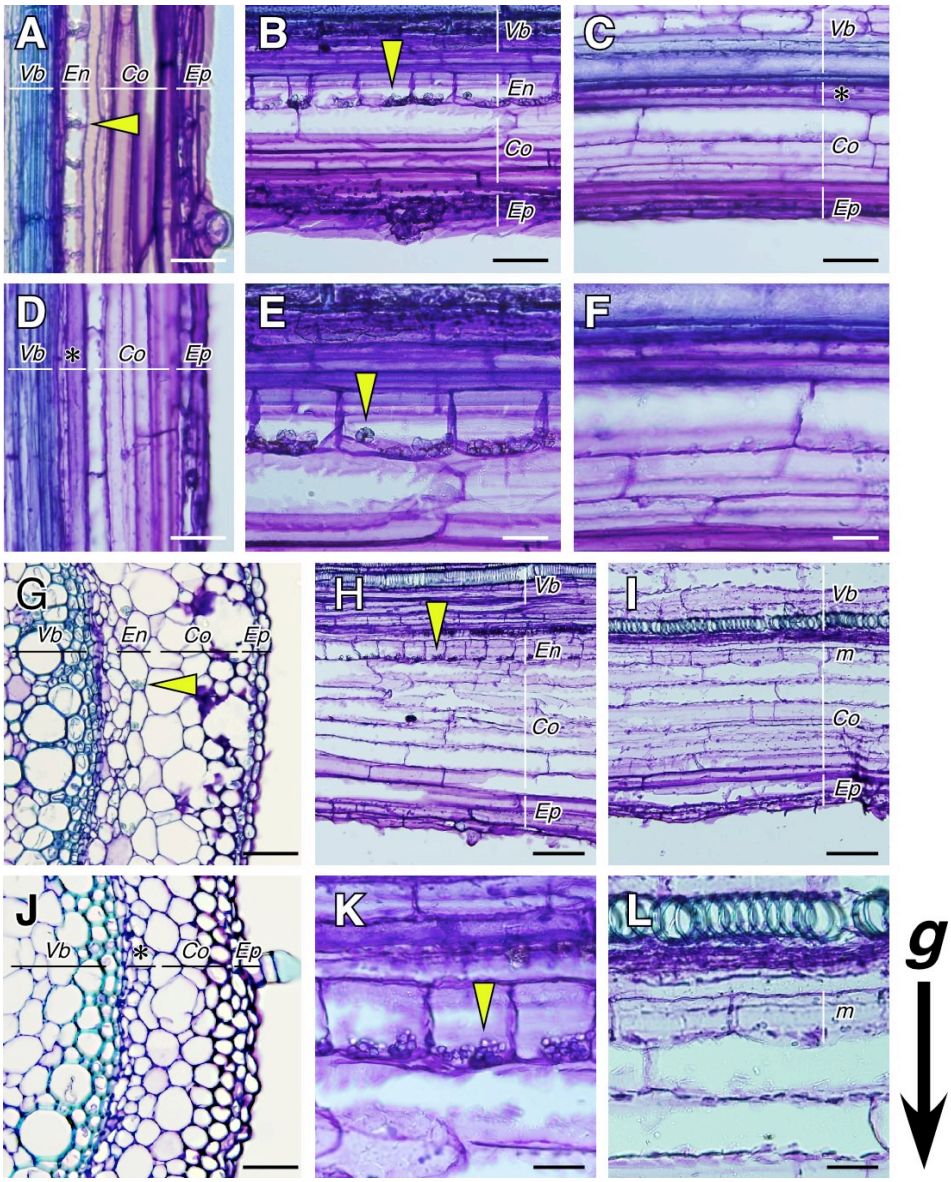


Fig. 1-7. Sections of morning glory stems and hypocotyls stained with toluidine blue O and I_2-KI solution. Longitudinal sections of vertically grown stems of WT (A) and *we2* (D) plants. Longitudinal sections of WT (B, E) and *we2* (C, F) stems that were re-oriented to a horizontal position for 3 h. Cross sections of stems from WT (G) and *we2* (J) plants that were placed in a horizontal position for 3 h. Longitudinal sections of hypocotyls from WT (H, K) and *we2* (I, L) plants that were placed in a horizontal position for 3 h. Arrowheads indicate sedimented amyloplasts. Arrow (*g*) indicates the direction of gravitational force. Asterisks indicate the region where endodermal cells would appear if they were present. *Ep*, epidermis; *Co*, cortex; *En*, endodermis; *Vb*, Vascular bundle; *m*, mutant cell layer. Scale bars: 50 µm in (A-D, G, J), 20 µm in (E, F, K, L), and 100 µm in (H, I).

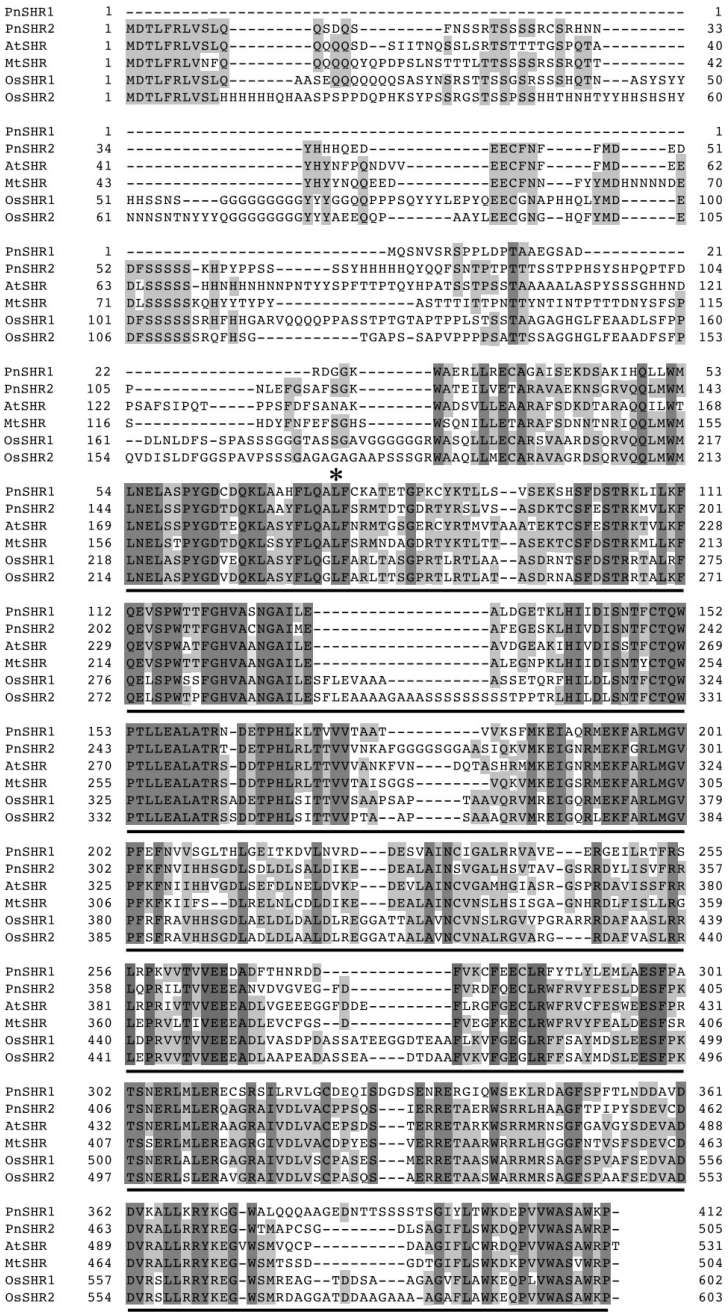


Fig. 1-8. Amino acid sequence alignment of SHR proteins. Identical amino acid residues are highlighted in dark gray, amino acid residues that are conserved in >50% of the protein sequences are highlighted in light gray. The conserved GRAS domain is underlined. The asterisk indicates the site of the mutation of *PnSHR1* in *we2* mutants. GenBank accession numbers: *Pharbitis nil* SHR1 (PnSHR1), AB330311, and SHR2 (PnSHR2), AB330312; *A. thaliana* SHR (AtSHR), AF233752; *Medicago truncatula* SHR (MtSHR), AC147000; *Oryza sativa* SHR1 (OsSHR1), AP003985, and SHR2 (OsSHR2), ABF96780.

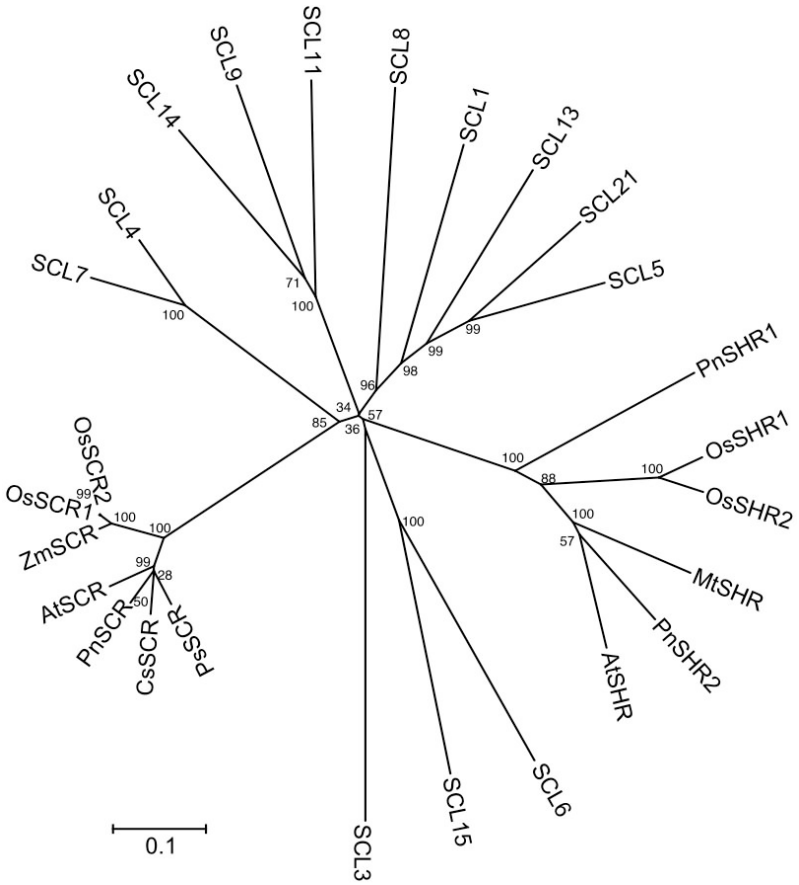


Fig. 1-9. Phylogenetic analysis of plant SHR and SCR family members. An unrooted phylogenetic tree was generated from multiple sequence alignments using ClustalW. The following sequences from the NCBI sequence database were used for the sequence alignment: SCL1 (AF210731), SCL3 (AF036301), SCL4 (AY062538), SCL5 (AF036302), SCL6 (AL161471), SCL7 (AL132979), SCL8 (AF036305), SCL9 (AF036306), SCL11 (AF036307), SCL13 (AF036308), SCL14 (AF036309), SCL15 (AK118497), SCL21 (AF210732), AtSCR (AB010700), CsSCR (AJ870307), PnSCR (AB200391), PsSCR (AB048714), OsSCR1 (NM001072149), OsSCR2 (AB180961), ZmSCR (AF263457). The alignment was analyzed by eye, and regions without a GRAS domain were removed. The phylogenetic tree was generated using the Neighbor Joining method and a distance matrix with corrections for multiple substitutions. MEGA3 (http://www.megasoftware.net/mega.html) was used to draw the tree. Numbers at the branch forks represent the bootstrap values, which are expressed as a percentage of 1,000 bootstraps, and a confidence limit for the sequence groupings.

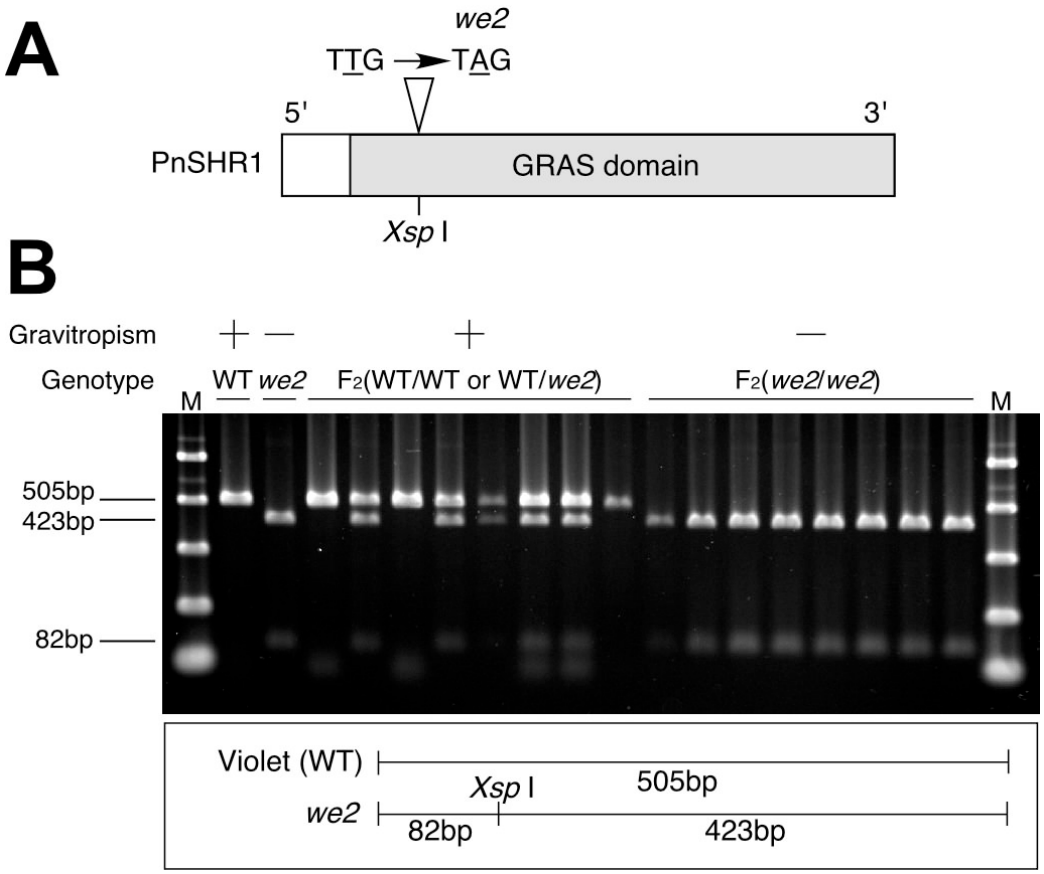


Fig. 1-10. Schematic illustration of the structures of *PnSHR1*, showing the site of the T-A transition in *we2* mutants (A). (B) Linkage analysis of agravitropism and *PnSHR1* mutation in *we2*. Dark-grown F_2 seedlings were grouped according to their gravitropic response, and *PnSHR1* was amplified by PCR from genomic DNA isolated from each F_2 seedling. Amplified PCR products were digested with *Xsp*I and analyzed by agarose gel electrophoresis. M, molecular weight marker (PCR marker; New England Biolabs, Beverly, MA, USA). The data from eight F_2 individuals of each phenotype are shown.

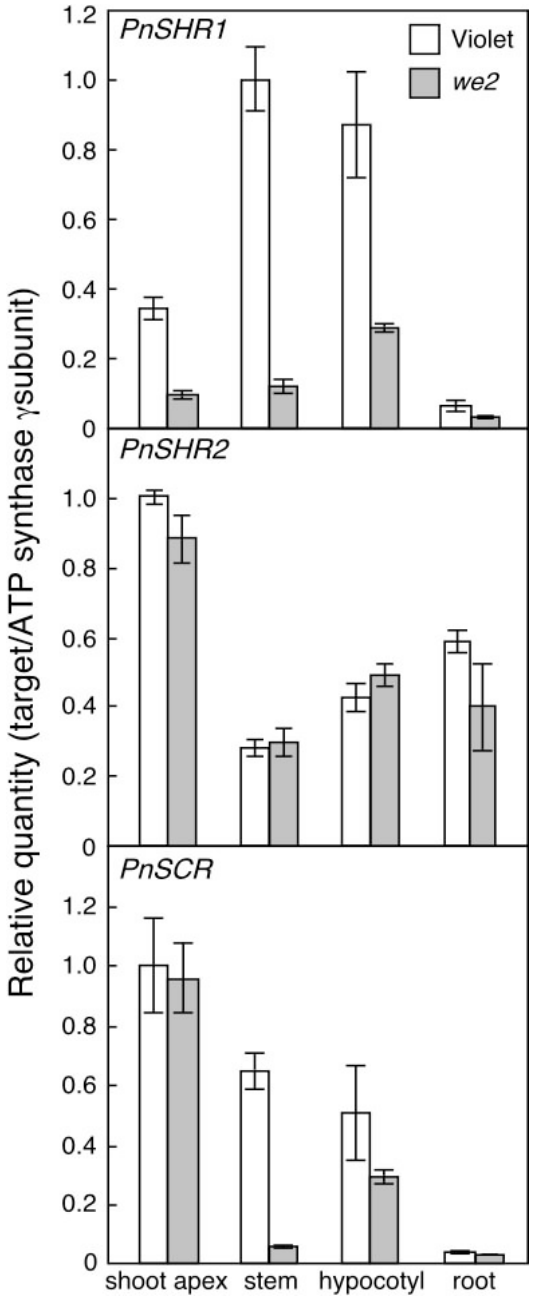


Fig. 1-11. Quantitative RT-PCR analysis of the expression of *PnSHR1*, *PnSHR2* and *PnSCR* in the organs of morning glory. The expression levels of each gene were normalized to the expression of the mitochondrial F_1F_0 ATP synthase γ-subunit, and relative expression levels are shown. Data represents the averages and standard deviation.

CHAPTER 2

Investigation of the relationship between shoot circumnutation and the graviresponse

This chapter is written on referring, in part, to following papers:

Kitazawa, D., Hatakeda, Y., Kamada, M., Fujii, N., Miyazawa, Y., Hoshino, A., Iida, S., Fukaki, H., Morita, M.T., Tasaka, M., Suge, H. and Takahashi, H. (2005) Shoot circumnutation and winding movements require gravisensing cells. *Proc. Natl. Acad. Sci. USA* 102: 18742–18747.

Kitazawa, D., Miyazawa, Y., Fujii, N., Nitasaka, E. and Takahashi, H. (2008) Characterization of a novel gravitropic mutant of morning glory, *weeping2*. *Adv. Space Res*. (in press, doi: 10.1016/j.asr.2007.10.029).

ABSTRACT

Circumnutation and winding movements are universal features of growing plants and improve plant viability despite their sessile nature. The gravity-dependence of these oscillatory movements has been a controversial matter for two centuries. To date, it remains unclear whether the endodermis-mediated graviresponse is indispensable for oscillatory movements. In order to obtain conclusive evidence that the graviresponse directly regulates oscillatory movements, I analyzed shoot circumnutation in multiple mutants of morning glory and *Arabidopsis*. Introducing wild-type *PnSCR* to *scr Arabidopsis* restored its defective circumnutation, demonstrating that the endodermis is solely required for proper circumnutation. Shoot circumnutation was impaired in *sgr2* and *sgr4/zig Arabidopsis* mutants, which have endodermal cell layers with abnormal amyloplast sedimentation, indicating that circumnutation requires amyloplast sedimentation in response to gravity. It was shown that *we* hypocotyls exhibit an impaired gravitropic response, but *we2* hypocotyls display a reduced gravitropic response. I investigated the correlation between the amplitudes of hypocotyl circumnutation and the gravitropic response. *we* hypocotyls did not exhibit an explicit circumnutation, whereas *we2* hypocotyls displayed a slight nutational movement. Taken together, these observations indicated that shoot circumnutation and winding movements solely require an endodermis-mediated graviresponse.

INTRODUCTION

Plant organs display oscillatory movements termed circumnutation (reviewed in Johnsson 1979, 1997). This movement is thought to help plant organs grow upward towards suitable environmental cues. Although the mechanism of circumnutation is not clear, the two-oscillator model proposed by Johnsson *et al.* (1999) has been widely accepted (see general introduction). In this model, the intrinsic oscillator causes plant organs to oscillate without gravity, and circumnutational activity is amplified by gravitropic-overshooting (Johnsson *et al*. 1999). The endogenous oscillator has been modeled as a growth wave traveling around the elongating organs that could be coupled with the oscillation of growth substances such as auxin and calcium (Johnsson *et al*. 1999). There has been no direct evidence to date for the involvement of the graviresponse as an external oscillator in circumnutation; this is controversial because hypocotyls of space-flown sunflowers exhibited circumnutation in microgravity, although the period and amplitude of the movements was smaller (Brown and Chapman 1984).

Circumnutation has diverse manifestations among different plant species. Generally, more substantial circumnutation can be observed in the vines and shoots of climbing plants (e.g., morning glory), which need to be anchored for support, compared to the shoots of non-climbing plants (Johnsson and Heathcote 1973, Johnsson 1977). In some plant species, such as morning glory, the stems wind along a support as the plant grows upward (Darwin 1876, Darwin and Darwin 1881). It is believed that circumnutation provides the motive power for the winding response of climbing plants, and is functionally related to these movements, but there is no direct evidence for the causal

relationship between circumnutation and the winding response. Thus, the detailed mechanisms explaining the relationships between the graviresponse, circumnutation, and the winding response are still obscure.

In order to obtain evidence that the graviresponse directly links oscillatory movements, I analyzed shoot circumnutation in multiple mutants of morning glory and *Arabidopsis*. As described in Chapter 1, *we* and *we2* mutants display agravitropism due to mutations in the *PnSCR* and *PnSHR1* genes of the *we* and *we2* plants, respectively, which confer defects in the normal differentiation of shoot-gravisensing endodermal cells in morning glory (Kitazawa *et al.* 2005, Kitazawa *et al*. 2008). Interestingly, it was previously found that *we* plants show defective circumnutation (Hatakeda *et al*. 2003). Thus, *weeping* mutants could be a powerful tool to investigate the role of endodermal cells during oscillatory movements.

In this chapter, I performed multiple molecular genetic analysis using morning glory and *Arabidopsis* mutants. First, I analyzed shoot circumnutation in *we2* mutants. Second, I investigated shoot circumnutation in transgenic *Arabidopsis* expressing wild-type *PnSCR* or *we*-type *PnSCR* (*PnSCRm*). Third, I examined shoot circumnutation in two agravitropic mutants of *Arabidopsis*, *sgr2* and *sgr4/zig*, which have endodermal cell layers with abnormal amyloplast sedimentation (see general introduction, Kato *et al.* 2002a, Morita *et al*. 2002). Fourth, I investigated circumnutation in the hypocotyls of wild-type and *weeping* morning glories.

MATERIALS AND METHODS

Plant materials and growth conditions

I used three strains of morning glory, Violet (WT), *we* and *we2* to investigate stem and hypocotyl circumnutation. Morning glory plants were cultivated as described in Chapter 1. I also used several strains of *Arabidopsis thaliana*. The wild-type ecotype Columbia and agravitropic mutants, *sgr1-1*/*scr-3*, *sgr2* and *sgr4*/*zig*, were used for the observation of shoot circumnutation. Transgenic *Arabidopsis* plants that had been introduced with *PnSCR, PnSCRm* or empty vector were also used in this study. *Arabidopsis* plants were grown as described in Chapter 1.

Analysis of nutational movement

In order to observe the nutational movements of morning glory shoots, potted plants with 30-cm-long stems and 6-cm-long hypocotyls (cotyledonary stage) were placed in a dark box. To observe the nutational movements of *Arabidopsis* shoots, potted plants were placed in a dark box when their inflorescence stems were about 9- to 10-cm long. Nutational movement was monitored by photographing the apical shoots with a digital camera (CAMEDIA E-10, Olympus) for 6 h at 5 min intervals. To avoid the occurrence of phototropic curvature of the inflorescence stems, plants were illuminated from the top by the programmed flash photography. The amplitude of the plant movements was measured from the images using image-analysis software (Image J; available on-line, http://rsb.info.nih.gov/ij/download.html). This also allowed the movement of shoot tips to be tracked by plotting their positions on *x*- and *y*-axes. Two-dimensional movements of shoot tips and the time required for shoot tips to turn

around once were defined as the ‘amplitude’ and ‘period’, respectively. Movements of at least five individual morning glory plants of each genotype were recorded and measured.

RESULTS

Nutational movement of we2 mutants

The shoots of *weeping* are defective in shoot circumnutation (Hatakeda *et al*. 2003). To determine whether *we2* mutants also lacked circumnutation, I analyzed shoot circumnutation in WT and *we2* mutants. In WT plants, the apical bud movement was circular as viewed from above (Fig. 2-1A). The amplitude and period of circumnutation was on average approximately 70 mm and 80-120 min, respectively, when the plants were approximately 30 cm in height (Fig. 2-1A). In contrast, *we2* mutant stems exhibited very small movements that were not circular (Fig. 2-1B, C). These results corroborated the results of the previous analysis of *we* mutants, and showed that the gravisensing endodermis is essential for circumnutation in shoots.

Function of PnSCR for shoot circumnutation

In order to test whether the mutation of PnSCR causes abnormal shoot circumnutation, I analyzed circumnutation in the wild-type, *scr,* and transgenic *Arabidopsis* plants used in Chapter 1.

In wild-type inflorescences, the amplitude and period of circumnutation was approximately 20 mm and 120 min, respectively. The apical bud movement of the wild type, as observed from the top, was circular or elliptical (Fig. 2-2A). In contrast, the *scr-3*

mutant and *scr-3*/Vector inflorescences exhibited very small movements that were not circular (Fig. 2-2E-H). In *scr-3/pAtSCR::PnSCR* inflorescences, circumnutation was restored to that of the wild type (Fig. 2-2B). In contrast, inflorescences on *scr-3/pAtSCR::PnSCRm* plants continued to exhibit severely reduced circumnutation (Fig. 2-2C, D).

Shoot circumnutation in sgr2 and sgr4/zig mutants

To determine whether endodermis-mediated gravisensing is indispensable for circumnutation, I analyzed shoot circumnutation in two agravitropic mutants of *Arabidopsis*, *sgr2* and *sgr4*/*zig*, which have endodermal cell layers with abnormal amyloplast sedimentation. In wild-type inflorescences, the apical bud movement, as observed from the top, was circular or elliptical similar to the above results (Fig. 2-3A). In contrast, inflorescence stems of *sgr2* and *sgr4/zig* mutants were defective in nutational movement (Fig. 2-3B-E).

Nutational movement in morning glory hypocotyls

As described in Chapter 1, *we* mutants completely lack a shoot gravitropic response, whereas the hypocotyls of *we2* mutants exhibit only a slight gravitropic curvature. If oscillatory movements mostly depend on the graviresponse, it would be expected that the impairment of circumnutation in *we* hypocotyls could be more severe than that of *we2*. To test this hypothesis, I compared the nutational movements of hypocotyls in *we* and *we2* mutants. The amplitudes of the oscillatory movements in WT

and *we2* were approximately 8.5 ± 3.2 mm and 3.9 ± 1.6 mm (mean ± SE), respectively (Fig. 2-4A, C, D, F), at the cotyledonary stage. In contrast, hypocotyls of *we* did not exhibit nutational movements (Fig. 2-4B, E). Thus, it appeared that the magnitude of nutational movements correlated with gravisensitivity in the hypocotyls of morning glory.

DISCUSSION

In order to obtain decisive proof that the graviresponse directly regulates oscillatory movement, I analyzed shoot circumnutation in multiple mutants of morning glory and *Arabidopsis*. Hatakeda *et al*. (2003) showed that stem circumnutation of *we* is impaired. I found that *we2* stems exhibited severely reduced circumnutation, corroborating that the endodermis is essential for circumnutation in the stem. Introducing wild-type *PnSCR* to the *scr Arabidopsis* mutant restored its circumnutation. This demonstrates that the *SCR* gene regulates circumnutation and gravitropism in both *Arabidopsis* and morning glory; thus, clearly linking these two processes. Shoot circumnutation in the *sgr2* and *sgr4/zig Arabidopsis* mutants, which have endodermal cell layers with abnormal amyloplast sedimentation, was severely reduced. This result indicated that oscillatory movements require proper endodermis with normal amyloplast sedimentation. The relationship between circumnutation and starch-granules has been previously reported by Hatakeda *et al.* (2003). Indeed, the *Arabidopsis* mutant, *phosphoglucomutase* (*pgm*), has a reduced gravitropism caused by a loss of starch granules (Kiss *et al*. 1989), and exhibits circumnutation that is smaller than that of the WT (Hatakeda *et al*. 2003). Taken together, my data strongly support the hypothesis that gravisensing and circumnutation are linked.

No nutational movement was observed in *we* hypocotyls that completely lack the gravitropic response, whereas *we2* hypocotyls, which display a reduced gravitropic response, showed weak nutational movement. Thus, the magnitude of the nutational movement correlated with gravisensitivity in the hypocotyls of morning glory.

These observations do not support the hypothesis that circumnutation is caused by an internal driving force, originally proposed by Darwin and Darwin (1881). Moreover, these observations contradict the report by Brown and co-workers that sunflower hypocotyls showed circumnutation even in microgravity in space (Brown *et al.* 1990). How do I interpret these two reports? One unsatisfying suggestion is that the differences are caused by differences between the plant species. In fact, the nutational direction of shoot circumnutation does differ among plant species. In brief, apical buds of morning glory display left-handed rotation whereas apical buds of *Arabidopsis* exhibit alternate rotation (Schuster and Engelmann 1997). It was noted that the magnitude of circumnutation in sunflower hypocotyls was smaller under microgravity conditions than that on the ground. In this spaceflight experiment, the seeds were germinated on Earth and thus the seedlings had sensed gravity before and during the launch. Thus, the oscillatory movement of the plant organs might have been established on the ground, and continued in orbit. In order to address the question of whether circumnutation occurs in the gravity-free conditions of space, seeds should be germinated in microgravity and nutational movements in the seedlings should be compared with corresponding controls in space. Based on the evidence to date, there is strong support for the theory that circumnutation requires a graviresponse (Kitazawa *et al*. 2005, Kitazawa *et al*. 2008).

Yoshihara and Iino (2006) reported that circumnutation in clinorotated rice coleoptiles disappears, and agravitropic *lazy* mutants show no circumnutation. However, Yoshihara and Iino (2005) also showed that red light entirely inhibits the circumnutation of rice coleoptiles without reducing their gravitropic responsiveness. Furthermore, the non-circumnutating *lazy* coleoptiles showed an almost wild-type level of gravitropic responsiveness in their upper half, although this was an active site of both gravitropism and circumnutation in wild-type coleoptiles (Yoshihara and Iino 2006). The authors therefore advocated that there are cases in which gravitropism and circumnutation is separated, and that circumnutation is not a simple consequence of the gravitropic response in rice coleoptiles, although it is obvious that the circumnutation of rice coleoptiles requires gravisensing processes. Based on my present studies, however, both gravisensing and the gravitropic response are linked to circumnutation in morning glory and *Arabidopsis* plants. Indeed, the conflicts between the conclusions of my study and those of Yoshihara and Iino (2005, 2006) might be caused by plant species differences, but in most cases, both gravisensing and the gravitropic response could be indispensable for circumnutation.

The widely accepted two-oscillator model that was proposed by Johnsson and co-workers defined that circumnutation is generated by both graviresponse-dependent and –independent oscillators. That is, a role for the graviresponse in circumnutation is the amplification of nutational movement caused by an internal oscillator such as auxin (Johnsson *et al*. 1999). The internal oscillator has been modeled as a growth wave traveling around the elongating organs (Johnsson *et al.* 1999). My study indicates that

the graviresponse acts as a trigger for generating nutational movement because the agravitropic stems of two *weeping* mutants did not exhibit nutational growth. The graviresponse might therefore primarily influence the traveling wave of the intrinsic oscillator such as auxin, and which could ultimately generate nutational movement.

The vine shoots of both *weeping* mutants completely lack the ability to wind around a support; therefore, the graviresponse might also be required for the winding response of the vine shoot in morning glory, a typical climbing plant. In classical observations, Darwin and Darwin (1881) proposed that both shoot circumnutation and the thigmomorphogenetic response due to the vine touching a support were important for plants to climb and to wind around a support. Since *we* mutants express normal touch responses (thigmomorphogenesis), it seems that shoot circumnutation plays a key role in the winding phenomenon and that the touch-response does not play a major role in the winding phenomenon. Alternatively, climbing plants may have a specialized touch-response in the shoot apex due to circumnutation. Nonetheless, it is true that gravitropism is genetically linked to both circumnutation and winding.

I have demonstrated that the endodermis-mediated graviresponse is indispensable for circumnutation and the winding response in morning glory. It is important to identify the factors responsible for these phenomena functioning downstream of gravisensing.

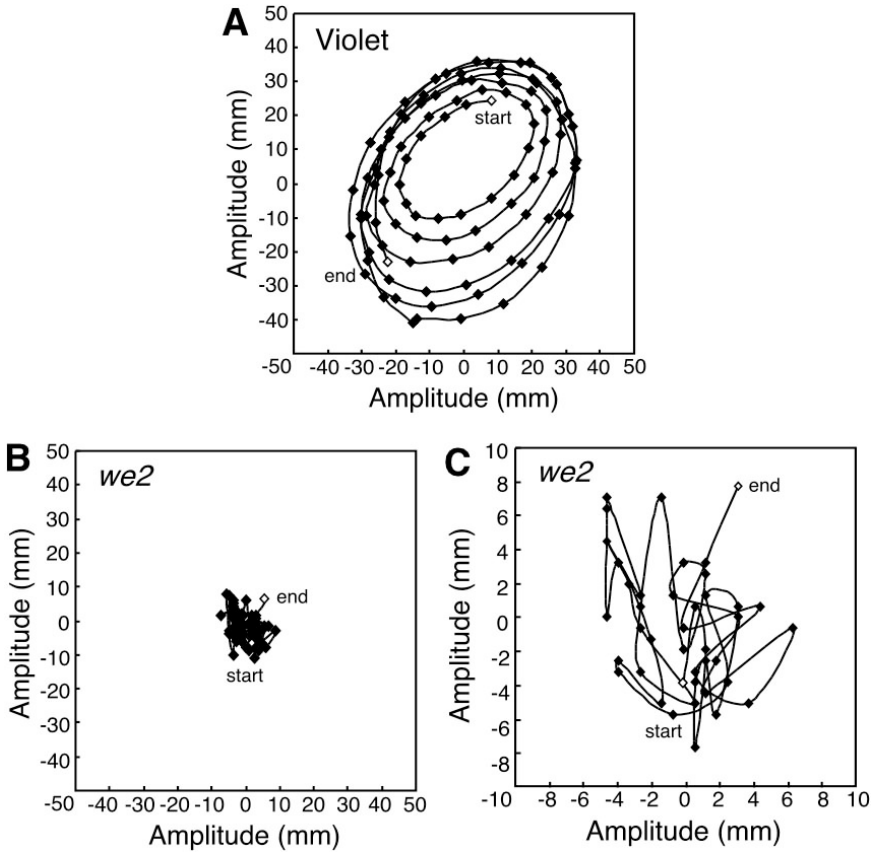


Fig. 2-1. Shoot circumnutation in WT and *we2* plants. Potted plants were placed in a dark box when their height reached approximately 30 cm. Movement of the shoot apex was recorded from the top. Circumnutation of WT (A) and *we2* mutants (B). (C) The data in (B) was re-plotted on a smaller scale (higher magnification). The data points corresponding to the start and the end of the period of observation are indicated by open squares.

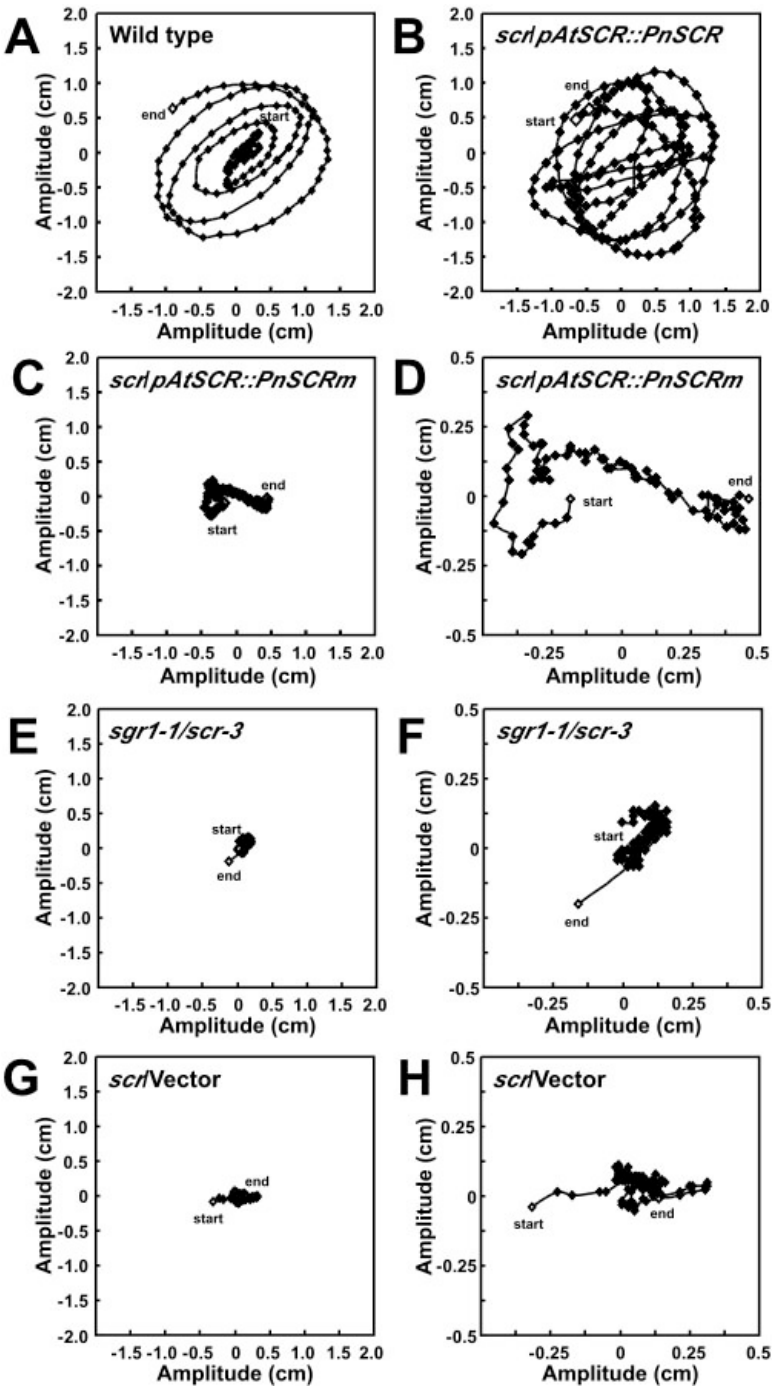


Fig. 2-2. The circumnutation of inflorescence stems in wild-type (WT), *scr,* and transformant *Arabidopsis*. Potted plants were placed in a dark box when their inflorescence lengths were approximately 9 to 10 cm. Shoot movement was observed from the top. (A) WT, (B) *scr*/*pAtSCR::PnSCR*, (C) *scr*/*pAtSCR::PnSCRm*, (E) *sgr1-1/scr-3*, (G) *scr*/Vector. D, F, and H show magnified images of C, E, and G, respectively. The start and the end of the movements are indicated by open squares.

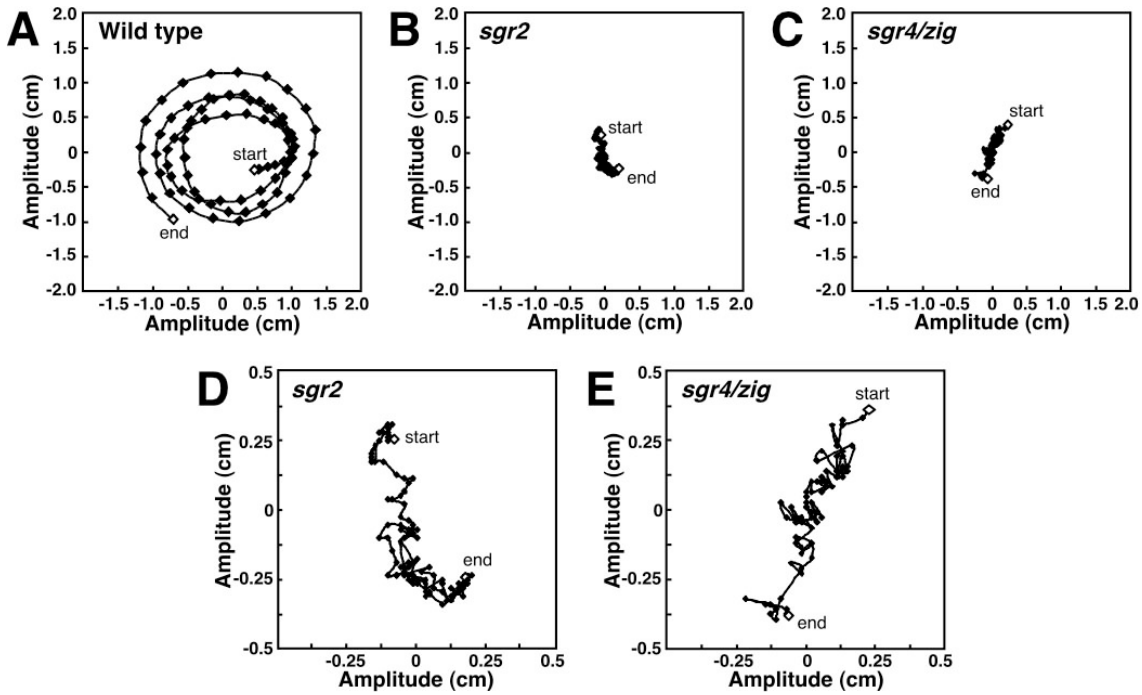


Fig. 2-3. Nutational movement in the inflorescence stems of the agravitropic mutants of *Arabidopsis*, *sgr2* and *sgr4*/*zig*. Potted plants were placed in a dark box when their inflorescence lengths were approximately 9 to 10 cm. Shoot movement was observed from the top. (A) Wild-type Columbia, (B) *sgr2*, (C) *sgr4/zig*. D and E show magnified images of B and C, respectively. The start and the end of the movements are indicated by open squares.

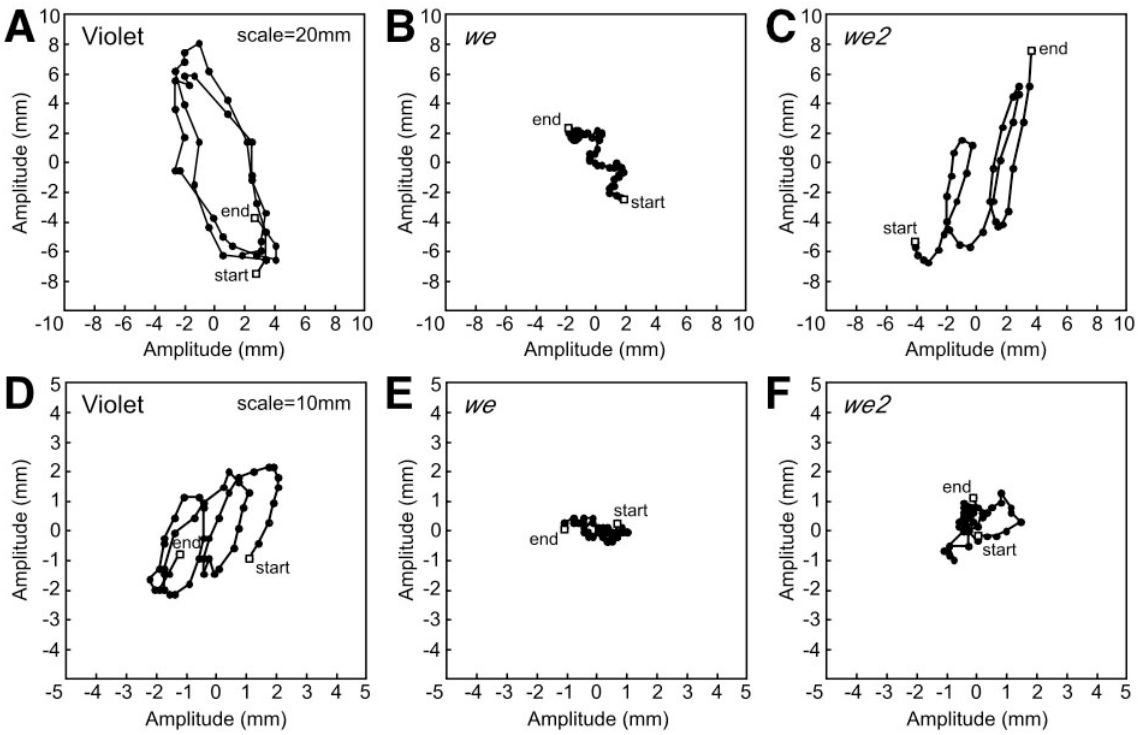


Fig. 2-4. Hypocotyl circumnutation in WT, *we* and *we2* plants. Potted plants with 6 cm long hypocotyls (cotyledonary stage) were placed in a dark box. Movement of the shoot apex was recorded from the top. Circumnutation of WT (A, D), *we* (B, E) and *we2* mutants (C, F). The data points corresponding to the start and the end of the period of observation are indicated by open squares.

CHAPTER 3

Verification of the involvement of the graviresponse in apical dominance and investigation of its mechanism

ABSTRACT

When the upper part of the main shoot of the Japanese morning glory is bent down, the axillary bud situated on the uppermost node of the bending region is released from apical dominance and elongates. Here, I demonstrate that this release of axillary buds from apical dominance is gravity-regulated. I utilized two agravitropic mutants of morning glory that are defective in gravisensing cell differentiation, *weeping* (*we*) and *weeping2* (*we2*). Bending the main shoots of either *we* or *we2* plants resulted in minimal elongation of their axillary buds. This aberration was genetically linked to the agravitropism phenotype of the *weeping* mutants, which implied that shoot bending-induced release from apical dominance required a graviresponse. Previous studies have shown that basipetal translocation of auxin from the apical bud inhibits axillary bud growth, whereas cytokinin promotes axillary bud outgrowth. I therefore compared the roles of auxin and cytokinin in bending- or decapitation-induced axillary bud growth. In the wild-type and *we* plants, decapitation increased cytokinin levels and reduced the auxin response. In contrast, shoot bending did not cause significant changes in either the cytokinin level or auxin response, suggesting that the mechanisms underlying gravity- and decapitation-regulated release from apical dominance are distinct and unique.

INTRODUCTION

Apical dominance is the central regulatory system for maintaining plant shoot architecture, wherein the growing apical shoot suppresses the growth of axillary buds on the axils of the leaves below it (reviewed in Cline 1991). As described in the general introduction of this thesis, this system may be influenced by gravity. If this hypothesis is accurate, the graviresponse could intimately control plant shoot architecture. I have succeeded in demonstrating that the graviresponse is required for shoot circumnutation and winding movements in morning glory, using *weeping* mutants that are defective in gravisensing cell differentiation (see Chapter 1, 2). I envisaged that *weeping* mutants might also be useful not only for verifying the role of the graviresponse in apical dominance but also for directly analyzing the relationship between the function of the endodermis and apical dominance.

The plant hormones auxin and cytokinin are thought to have major roles in controlling apical dominance; with axillary bud growth being inhibited by the basipetal translocation of auxin but promoted by cytokinin (see the general introduction). Following decapitation of the shoot apex, auxin levels in the stem decrease, cytokinin levels increase, and the axillary buds elongate. Furthermore, direct application of cytokinin to axillary buds of intact plants is sufficient to promote their outgrowth, suggesting a preferential role for cytokinin in apical dominance (Pillay and Railton 1983, Cline et al. 1997).

Apical dominance is plastic in its response to various environmental cues such as light and nutrient conditions (Snowden and Napoli 2003), and may also be affected by

the influence of gravity, through the graviresponse. Bending the upper part of the main shoot releases the bending-region axillary bud from apical dominance and initiates elongation of the bud; this release is prevented by clinorotation of the bent plants (Prasad and Cline 1987). These results suggest that the release from apical dominance in morning glory is due, at least in part, to gravistimulation, although there is no evidence to explain the mechanism by which this occurs. It has been proposed that perhaps the plant hormone auxin mediates the influence of gravity on apical dominance; however, no change in auxin distribution during shoot bending was observed when the dynamics of endogenous auxin were examined via mass spectrometry (Prasad *et al.* 1993). In addition, there have been no reports delineating a role for cytokinin in axillary bud growth induced by shoot bending. It is therefore unclear whether shoot bending-induced release from apical dominance is governed by the same mechanisms as decapitation-induced release.

As described in Chapter 1, the agravitopic morning glory mutants, *we* and *we2*, have mutations in the *PnSCR* and *PnSHR1* genes, respectively, which confer defects in the normal differentiation of shoot-gravisensing endodermal cells (Kitazawa *et al.* 2005, Kitazawa *et al*. 2008). The shoots of these mutants lack a graviresponse, allowing the extraction of plant graviresponse phenotypes and genetic analyses through comparisons between *we*, *we2*, and wild-type plants. Morning glory is characterized as an absolute short-day plant, which is advantageous for the study of apical dominance because it enables researchers to carry out experiments in the vegetative stage without the floral transition of meristems. Rosette plant species such as *Arabidopsis thaliana* are

unsuitable for this study since its stem hardly elongates in the vegetative stage.

In this chapter, I first verified the gravity-regulated release of axillary buds from apical dominance in the Japanese morning glory by examining whether bending the main shoots of *weeping* strains caused an outgrowth of the axillary buds. I then investigated the involvement of the plant hormones auxin and cytokinin in the two modes of axillary bud outgrowth, bending- and decapitation-induced release from apical dominance.

MATERIALS AND METHODS

Plant materials and growth conditions

Morning glory plants, wild-type Violet, *weeping* and *weeping2* were cultivated as described in Chapter 1.

Shoot bending

For shoot-bending experiments, the internode above node 6 was gently bent down so that the upper part of the shoot was inverted when plants were four weeks old. The length of the axillary bud on node 6 (uppermost node) was monitored every day. Twelve individual plants were examined for each morning glory strain. Linkage analysis of F_2 plants between the gravitropic response and axillary bud growth due to shoot bending was performed as follows: the *we* and *we2* mutants were crossed with Violet, and the resulting F_1 plants were self-pollinated to generate the F_2 population. Twenty-five individual F_2 plants showing a gravitropic or agravitropic response were cultivated and

subjected to the shoot bending treatment when plants were four weeks old. Ten days after the bending treatment began, I measured the length of the axillary buds on the uppermost nodes.

Decapitation

For decapitation studies, two–week-old plants were decapitated, lanolin paste (lanolin-dehydrate; Wako) containing 0.1% (w/v) IAA (indole-3-acetic acid; Wako) was applied to the cut surface of the decapitated stem and the plants were grown for six days. Plants without decapitation and decapitated plants to which lanolin paste without IAA was applied were also grown. The length of the axillary buds was monitored every day, and lanolin paste was renewed every other day. Twenty-four individuals were examined in each experiment.

RNA isolation and the cloning of full-length PnIAA1 and PnIPT1 cDNAs

Total RNA was extracted from the stems of two-week-old greenhouse-grown WT plants, using an RNeasy Plant Mini kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized using a ReverTra Ace α kit (TOYOBO) following the supplier's instructions. The EST database of the Japanese morning glory contained one sequence that was likely to be transcribed from a gene homologous to *Aux/IAA* and two sequences that were likely to be transcribed from genes homologous to *ATP/ADP IPT*. These EST sequences contained full-length cDNA sequences of the corresponding genes. These sequences were amplified using the following gene specific

primers: 5’-AATGACGGCGAAGCGGTGAA-3’ (forward) and 5’-GGATCAGAAGCCATTGGACT-3’ (reverse) for *PnIAA1*, and 5’-TAACAAGGTCACCGACGAGG-3’ (forward) and 5’-AATGGCGTCTTCCAGTAGCC-3’ (reverse) for *PnIPT1.* Amplified cDNA fragments were then cloned into the pGEM-T Easy vector (Promega). DNA sequences were determined using an ABI 310 Genetic Analyzer (Applied Biosystems) according to the standard procedures.

Quantitative reverse-transcription (RT)-PCR analysis

First-strand cDNA was synthesized as described above. Quantitative real-time PCR was performed on a MyiQ single-color real-time PCR detection system using an iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer’s instructions. The target gene expression was normalized to mitochondrial F_1F_0 ATP synthase γ-subunit expression. PCR was carried out using the following program: 120 s at 95°C, 45 cycles of 10 s at 95°C, 30 s at 55°C (for *PnIAA1* and *ATP synthase*) or 30 s at 57°C (for *PnIPT1*), 45 s at 72°C, followed by melting curve analysis. Calibration was performed using a template cDNA fragment cloned in a plasmid. The following primers were used for the PCR amplification: PnIAA1 F1 (5’-AATGACGGCGAAGCGGTGAA-3’) and PnIAA1 R1 (5’-GGATCAGAAGCCATTGGACT-3’) for *PnIAA1*, PnIPT1 F2 (5’-TAACAAGGTCACCGACGAGG-3’) and PnIPT1 R2 (5’-AATGGCGTCTTCCAGTAGCC-3’) for *PnIPT1*, ATPS F (5’-CAGTGGATCCTGATGACATCCTTAAAAATG-3’) and ATPS R

(5'-CTTCCTCGAGTTTTATTTCATCACCATCAG-3') for *ATP synthase γ-subunit*.

Measurement of trans-zeatin riboside (t-ZR)

The *t*-ZR standard was purchased from the Sigma Chemical Co., and was prepared via a dilution series with absolute methanol to generate a standard curve. Samples of morning glory were frozen once with liquid nitrogen, and then homogenized in absolute methanol. After centrifugation of the homogenate, the supernatant was collected as a crude extract. I used a commercial competitive ELISA kit (*trans*-zeatin riboside immunoassay detection kit; Sigma) to perform the assay. The crude extracts were preincubated in wells for 3 h at 4°C and then placed in an incubator at 37°C for 1 h. Resultant products of the enzymatic reactions were quantified spectroscopically at 405 nm. The detailed procedure was as per the manufacturer's instructions.

RESULTS

Effect of shoot bending on axillary bud growth

Upon bending an upright morning glory shoot downward, the axillary bud situated on the uppermost node is released from apical dominance and begins to elongate; this response is obliterated by clinorotation (Prasad and Cline 1987). These results suggest that the bending-induced outgrowth of the axillary bud depends on the graviresponse. I verified this hypothesis using the agravitropic *weeping* mutants of morning glory. The axillary buds located at nodes 1 through 3 of four-week-old WT, *we,* and *we2* plants grown under conventional conditions were approximately 2 mm in length. There was no

statistical difference between the lengths of these axillary buds. I performed the “shoot bending treatment,” according to the procedure described by Cline *et al*. (1983) using WT, *we*, and *we2* morning glory shoots. When the main shoots of four-week-old plants were bent down above node 6, the axillary bud on node 6 (the uppermost node) began to elongate in WT plants, whereas no outgrowth of the axillary bud was observed in either the *we* or *we2* mutants (Fig. 3-1A, B). To investigate whether this aberrant response of the axillary buds was genetically linked to the agravitropism of these mutants, I performed a linkage analysis using the F_2 generation. As shown in Fig. 3-2, all gravitropic F_2 generation plants responded to shoot bending by an outgrowth of the axillary buds on their uppermost nodes. In contrast, elongation was not observed in any of the agravitropic F_2 generation plants. These results clearly demonstrated the hypothesis that graviresponse is involved in apical dominance.

Effect of decapitation on axillary bud growth

Classical studies have shown that pinching off the shoot tip (decapitation) induces the outgrowth of the axillary buds in many plant species, a process that is often used for the study of apical dominance (reviewed in Cline 1991). In order to examine whether the endodermis-mediated graviresponse also affects the mechanisms regulating decapitation-induced axillary bud outgrowth, I decapitated the shoots above node 1 of two-week-old WT, *we*, and *we2* morning glory plants. I also investigated the response of these decapitated plants to the application of exogenous auxin to the stump, and determined whether the presence or absence of an endodermis affected apical

dominance-associated basipetal auxin translocation.

Decapitation of WT plants caused a rapid release of the axillary buds from apical dominance (Fig. 3-3, upper panel). Likewise, decapitation induced outgrowth of the axillary buds in both *we* and *we2* plants (Fig. 3-3, middle and lower panel). Exogenous IAA applied to the cut surface of the decapitated stem inhibited axillary bud outgrowth in WT, *we*, and *we2* plants (Fig. 3-3), suggesting that abnormal development of the endodermis does not affect apical dominance-related auxin translocation. These results further suggest that the graviresponse does not influence decapitation-induced axillary bud growth.

Molecular identification of auxin and cytokinin reporter genes

The molecular mechanisms underlying shoot bending-induced release from apical dominance remain unclear. Auxin and cytokinin are known to play a role in decapitation-induced release, and it is therefore important to clarify whether the dynamics of these hormones are also involved in bending-induced release. In this study, I examined the involvement of auxin and cytokinin in bending- and decapitation-induced axillary bud growth. An auxin-inducible gene and a cytokinin-biosynthetic gene were adopted as molecular markers.

Aux/IAA is a well-characterized family of auxin-inducible genes (Tiwari *et al.* 2004). I isolated *Aux/IAA* homologous genes from the Japanese morning glory as follows. Using a PCR-based strategy (see Materials and Methods), I isolated a cDNA clone homologous to *Aux/IAA* from the Japanese morning glory cv. Violet, based on the

sequence information in the expressed sequence tag (EST) of the Japanese morning glory cv. TKS (Morita *et al*. 2006a). The predicted polypeptide of the obtained cDNA sequence contains domains I, II, III and IV, which are highly conserved among Aux/IAA protein families (Tiwari *et al*. 2004). I then verified whether the corresponding gene was responsive to auxin using a stem section of morning glory, according to a method described previously (Fujii *et al*. 2000). The expression of the gene in the excised sections of the WT plant was reduced by auxin starvation, while subsequent treatment of the sections with exogenous IAA elevated its expression in a dose-dependent manner (Fig. 3-4A). These data indicate that cDNA isolated from the stem of the Japanese morning glory plant is transcribed from a gene belonging to the auxin-responsive *Aux/IAA* family. I named this gene *PnIAA1* (GenBank accession no. AB371299).

It has been previously reported that the cytokinin biosynthetic gene *isopentenyltransferase* (*IPT*) contributes to decapitation-induced axillary bud growth in the pea plant (*Pisum sativum* L.) (Tanaka *et al*. 2006). Following decapitation, levels of cytokinin and *PsIPT1* and *PsIPT2* mRNA were markedly increased in the nodal stem. The expression of these genes was repressed by the application of IAA.

I then searched for *IPT* homologous genes in the morning glory EST database. In *Arabidopsis*, the IPT family is known for its diverse functions (Miyawaki *et al*. 2006). ATP/ADP IPT and their homologs, AtIPT1 and AtIPT3–AtIPT8 are *Arabidopsis* enzymes that catalyze the first step of cytokinin biosynthesis (Kakimoto 2003, Miyawaki *et al*. 2004). AtIPT2 and AtIPT9 are thought to catalyze the isopentenylation of tRNA, and are probably not involved in cytokinin biosynthesis (Golovko *et al*. 2002). I performed a

phylogenetic analysis of members of the IPT family in various plants based upon the information in the EST database (Fig. 3-4B). Since two EST sequences were likely to be transcribed from genes that belong to the cytokinin biosynthetic IPT family, I used a PCR-based strategy to isolate the corresponding cDNAs from the WT morning glory (see Materials and Methods). The cDNAs that were obtained for the two *IPT*-homologous genes, *PnIPT1* and *PnIPT2*, encode putative proteins with sequence identities of 50% and 52%, respectively, to PsIPT1; and 44% and 49%, respectively, to PsIPT2. I therefore designated these *IPT*-homologous genes *PnIPT1* and *PnIPT2*, respectively (GenBank accession nos. AB371300 and AB371301, respectively).

Effect of decapitation on the expression of PnIAA1 and PnIPT1

To examine whether *PnIAA1* and *PnIPTs* respond to decapitation, I studied their expression patterns in the first node of morning glory shoots before and after decapitation. The mRNA level of *PnIAA1* began to decrease 1 h after decapitation, and reached a minimum 3 h after decapitation (Fig. 3-5A). In contrast, the mRNA level of *PnIPT1* began to increase 1 h after decapitation, reached a maximum level after 3 h, and then began to decrease 6 h after decapitation (Fig. 3-5B). This expression pattern was similar to that of *PsIPT2* reported previously (Tanaka *et al*. 2006). In the current study, the mRNA level of *PnIPT2* was low, and did not show an explicit expression pattern in response to decapitation (data not shown).

In order to examine the correlation between the expression of *PnIPT1* and the level of endogenous cytokinin, I used an ELISA method to quantify *trans*-zeatin riboside (*t*-ZR),

one of the active cytokinin compounds, before and after decapitation (Fig. 3-5C) (Weiler 1980, Weiler 1984). Decapitation induced a marked increase in the level of *t*-ZR in the node situated below the decapitated stump in the WT plant, implying a correlation between *PnIPT1* expression and the level of endogenous cytokinin. I therefore concluded that the *PnIPT1* gene could be used as a reporter for the analysis of cytokinin dynamics in the regulation of apical dominance.

Effect of auxin application on the expression of PnIAA1 and PnIPT1

To elucidate the relationship between basipetal auxin transport and the expression of *PnIPT1*, I investigated *PnIAA1* and *PnIPT1* expression in the node by applying IAA to the stump after decapitation. Furthermore, to examine whether decapitation-induced release from apical dominance is regulated by the same mechanisms in WT and *we* plants, I analyzed the expression of the marker genes in *we*. Immediately following decapitation of WT or *we* plants, lanolin with or without IAA was applied to their stumps. Three hours after treatment, the level of *PnIAA1* transcripts had decreased in WT and *we* plants treated with lanolin alone, and had increased in plants treated with IAA (Fig. 3-6A). This suggests that removal of the shoot tip (auxin source) causes a deprivation in the available endogenous auxin in the node; and that the IAA applied to the stump basipetally translocates from the stump to the node. In contrast, levels of the *PnIPT1* transcript were increased by lanolin alone, but not by IAA, in WT plants (Fig. 3-6B), strongly suggesting that *PnIPT1* transcription is negatively controlled by the basipetal transport of auxin in the stem. In *we* mutants, *PnIAA1* and *PnIPT1* expression was

similar to that observed in WT plants (Fig. 3-6A, B). This result demonstrates that the release from apical dominance in WT or *we* plants is decapitation-induced, and that this mechanism of release mediates auxin and cytokinin dynamics.

Effect of shoot bending on the expression of PnIAA1 and PnIPT1

I next examined the involvement of auxin and cytokinin in the gravity-regulated growth of the axillary bud. I analyzed *PnIAA1* and *PnIPT1* expression in node 6 at the bending region (uppermost node) or in the corresponding node (node 6) of control upright WT and *we* plants. Surprisingly, shoot bending resulted in minor changes in the mRNA levels of *PnIAA1* and *PnIPT1* in WT plants 72 h after bending treatment (Fig. 3-7A). Likewise, the level of *PnIAA1* transcripts in *we* mutants did not display significant fluctuations, nor were there significant differences between *we* and WT *PnIAA1* transcripts (Fig. 3-7A). The level of *PnIPT1* in *weeping* did not differ from that in the WT 24 h after shoot bending, although this level decreased in the WT at subsequent times (Fig. 3-7A). To confirm these results, I used an ELISA method to measure the endogenous cytokinin content in the uppermost node of the bent plants. In contrast with the results of the decapitation experiments, endogenous *t*-ZR levels did not significantly increase in the uppermost node within 24 h of bending in both WT and *we* plants (Fig. 3-7B). This result was consistent with the expression pattern of *PnIPT1*. These observations imply that dramatic changes in basipetal auxin transport and cytokinin biosynthesis in the uppermost node are not involved in the bending-induced release from apical dominance. Consequently, I conclude from these data that the mechanism

controlling gravity-regulated release from apical dominance is unique from that of decapitation-induced release, and possibly involves a novel pathway that requires neither a reduction in basipetal auxin transport nor cytokinin biosynthesis.

DISCUSSION

My results in this study using agravitropic shoots of morning glory demonstrate that the graviresponse plays an important role in the bending-induced release of axillary buds from apical dominance. Shoot bending stimulated the outgrowth of the axillary bud situated on the uppermost node in WT but not in *weeping* plants. Interestingly, these *weeping* mutants showed a normal response to decapitation, stimulating outgrowth of axillary buds just below the cut stump. Auxin applied to the stump suppressed the decapitation-induced release of axillary buds from apical dominance in both WT and *weeping* plants. These results suggest that the graviresponse mediates bending-induced release from apical dominance but not the decapitation-induced response. Thus, the mechanisms for the two modes of axillary bud growth may differ from one another.

Auxin and cytokinin are thought to be key regulators of apical dominance. Basipetal translocation of auxin from the shoot apex inhibits axillary bud growth, while cytokinin promotes this process. In many plant species, the direct application of cytokinin to axillary buds promotes their outgrowth, and endogenous cytokinin levels rise in and around axillary buds during growth initiation (Li *et al*. 1995, Turnbull *et al*. 1997, Emery *et al*. 1998). There is unequivocal evidence for cytokinin biosynthesis in shoots (Nordström *et al*. 2004). To date, cytokinin is the only plant hormone known to induce the outgrowth

of axillary buds. Tanaka *et al*. (2006) proposed that one role of auxin is to repress *IPT* gene expression; that is, local biosynthesis of cytokinin in the nodal stem is negatively regulated by auxin through the control of *IPT* expression in apical dominance. In agreement with this hypothesis, *PnIPT1* gene expression was up-regulated by removal of the auxin source, and was repressed by exogenous IAA application to the cut stump in the morning glory. This suggests that the mechanism underlying the relationship between auxin and cytokinin in the regulation of apical dominance is universal among various plant species.

When the upper shoot of the WT plant is bent down, the axillary bud on the uppermost node begins to grow out within 24-36 h (Cline and Riley 1984). The kinetics of axillary bud growth induced by shoot bending are similar to those induced by decapitation (Figs. 3-1, 3-3). It would therefore be possible that bending-induced release from apical dominance is also mediated by auxin and cytokinin. Surprisingly, shoot bending (gravistimulation) did not affect auxin and cytokinin dynamics, despite being accompanied by axillary bud outgrowth. Prasad *et al.* (1993) reported that shoot bending treatment did not significantly cause a reduction in basipetal auxin transport from the shoot apex. The expression of an auxin-inducible gene (*PnIAA1*) hardly changed during bending-induced release from apical dominance (Fig. 3-7A), supporting this observation. In addition, I measured the basipetal auxin transport activity using ^{3}H-labeled IAA in WT and *we* plants. My results showed that basipetal transport activity of exogenously applied ^{3}H-IAA in *we* did not differ from that of WT (Table 3-1). This observation also suggests that the gravity-regulated release of axillary buds from apical dominance is not

related to basipetal auxin transport. To my knowledge, this study is the first report investigating cytokinin dynamics during bending-induced release from apical dominance. The expression of *PnIPT1* and the level of endogenous *t*-ZR did not change significantly during shoot bending, in a sharp contrast to the changes that were observed in the decapitation-induced response.

Taking all of this information into consideration, I conclude that changes in basipetal auxin transport and *de novo* cytokinin biosynthesis in the uppermost node are not necessarily required for gravity-regulated axillary bud growth; this implies the existence of a novel system that functions in the regulation of axillary bud growth.

One key to elucidating the mechanism regulating bending-induced release from apical dominance may be hidden in the graviresponse cascade that is mediated by the gravisensing endodermal cells. The agravitropic-*weeping* mutants used here lack endodermal cells (Kitazawa *et al*. 2005, Kitazawa *et al*. 2008). Since *weeping* mutants do not respond to shoot bending and respond normally to decapitation, it is clear that endodermal cells are required specifically for the bending-induced release from apical dominance. The present study constructed an experimental system using agravitropic morning glory as a useful tool for the study of the gravity-regulated release from apical dominance. A novel pathway regulating axillary bud growth, which does not include auxin and cytokinin dynamics, needs to be clarified. Another intriguing question in shoot bending-induced release from apical dominance is how plants recognize the highest position of the axillary meristem, and how they direct the buds located in these nodes to initiate growth of a new apical meristem. Gravity-regulated axillary bud growth might be

an informative system for the study of plant science.

Table 3-1. Basipetal transport activity of IAA in Violet (WT) and *we* hypocotyls

	Upright (cpm)	Inverted	Horizontal
Violet	2039.4 ± 142.8	1931.6 ± 192.9	1950.4 ± 142.4
we	2173.6 ± 126.7	2280.7 ± 118.8	2466.4 ± 127.1

A 20-mm-long sub-apical (just below the hook) section of hypocotyls from 72-h-old etiolated seedlings were excised and placed in a vertical position in a 20-ml scintillation counting vial so that the basal end of the section was inserted into a 5 mm deep agarose (0.9%) plate. A 1.5% agarose block (2×2×2 mm) containing approximately 14,000 dpm 3-[5(n)-^{3}H]indolylacetic acid ([^{3}H]IAA; Amersham Pharmacia Biotech UK, Buckinghamshire, UK) dissolved in 5 mM potassium phosphate buffer at pH 5.5 was placed on the apical cut end of the section. For the measurement of IAA basipetal transport, vials containing hypocotyl sections were kept in either an upright, inverted or horizontal position for 5 h. After incubation, the section was cut into the distal and the proximal portions. The proximal portion of the section together with the receiver agarose plate was enclosed, and 5 ml of liquid scintillation cocktail (Ultima Gold; Packard Instrument Co., Meriden, CT, USA) was placed in the scintillation counting vials. After overnight extraction at room temperature, the ^{3}H-label of the entire phase of the uniformly mixed sample was measured in a Beckman multi-purpose scintillation counter (LS6500; Beckman Coulter Inc, Palo Alto, CA, USA). Data are the means ± SE.

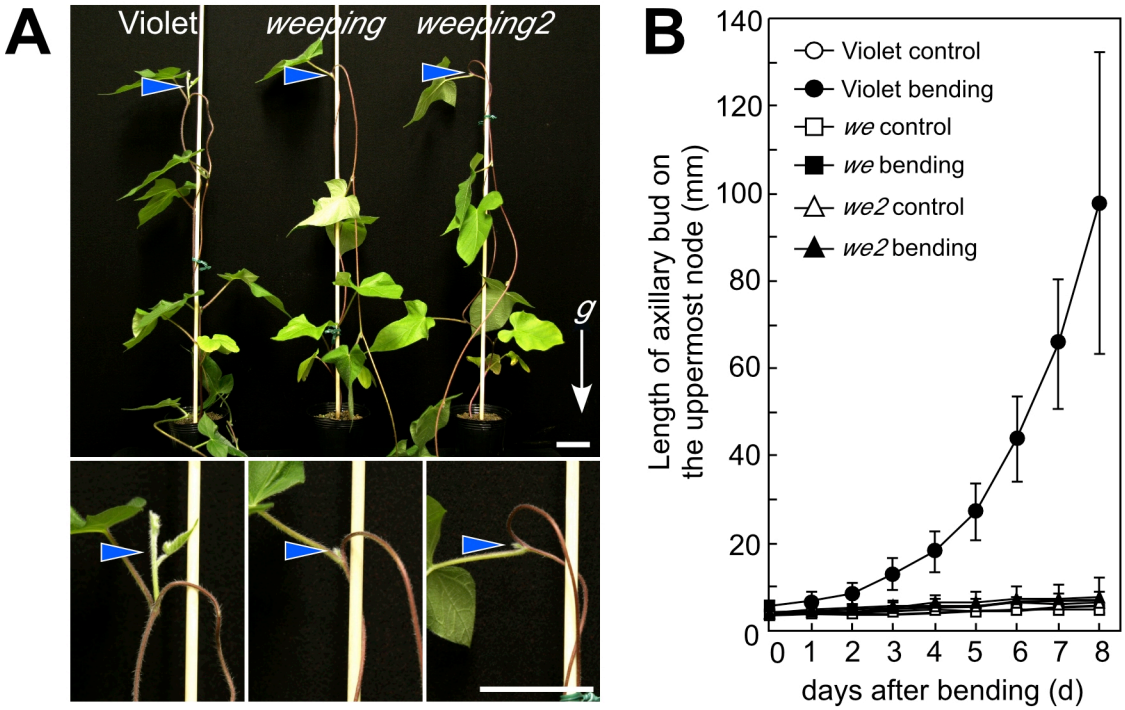


Fig. 3-1. Shoot-bending treatment in Violet and *weeping* mutants. (A) Photographs of the bent plants (upper panel) and magnification of one of their axillary buds on the uppermost node in the bending region (lower panel). Arrowheads indicate the axillary bud on the uppermost node. The arrow (*g*) indicates the orientation of gravity. Bars = 5 cm. (B) Measurement of the axillary bud length on the uppermost node. After bending treatment, the length of the axillary bud on the uppermost node was measured everyday. Data represent the means of twelve individuals. Vertical bars indicate ± SD. Open circles, Violet without bending treatment; closed circles, Violet with bending treatment; open squares, *weeping* without bending treatment; closed squares, *weeping* with bending treatment; open triangles, *weeping2* without bending treatment; closed triangles, *weeping2* with bending treatment.

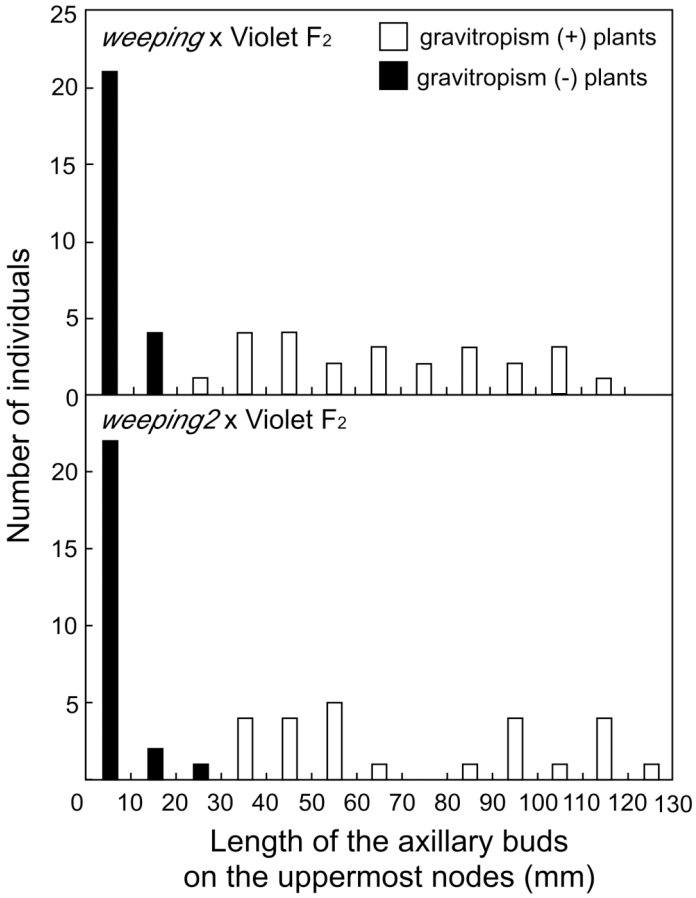


Fig. 3-2. F_2 linkage analysis between the gravitropic response and axillary bud growth. Ten days after shoot bending treatment, the axillary buds on the uppermost node were measured. The distribution of the length of the axillary buds on the uppermost node of F_2 plants is shown: *we* mutant F_2 segregants (upper panel), *we2* mutant F_2 segregants (lower panel). Open column, F_2 generation showing proper gravitropism; closed column, F_2 generation showing abnormal gravitropism. Twenty-five individual F_2 plants showing a gravitropic or agravitropic response were tested.

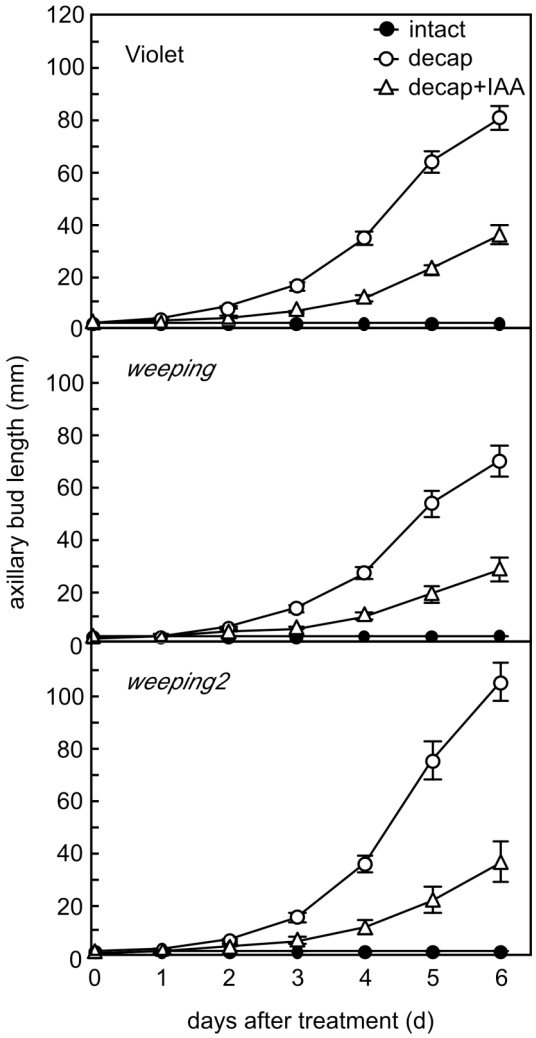


Fig. 3-3. Effects of decapitation and auxin application to the stump on axillary bud growth in Violet and *weeping* mutants. Two-week-old plants were decapitated and lanolin paste containing 0.1% (w/v) IAA was applied to the cut surface of the decapitated stem. Plants without decapitation and decapitated plants to which lanolin paste without IAA was applied (mock treatment) were also grown as controls. Axillary bud growth was monitored everyday, and lanolin paste was renewed every other day. The length of the axillary buds in twenty-four individuals was examined in each experiment. Data are the means ± SD. Closed circles, plants without decapitation; open circles, decapitated plants with mock treatment; open triangles, decapitated plants with IAA application to the stump.

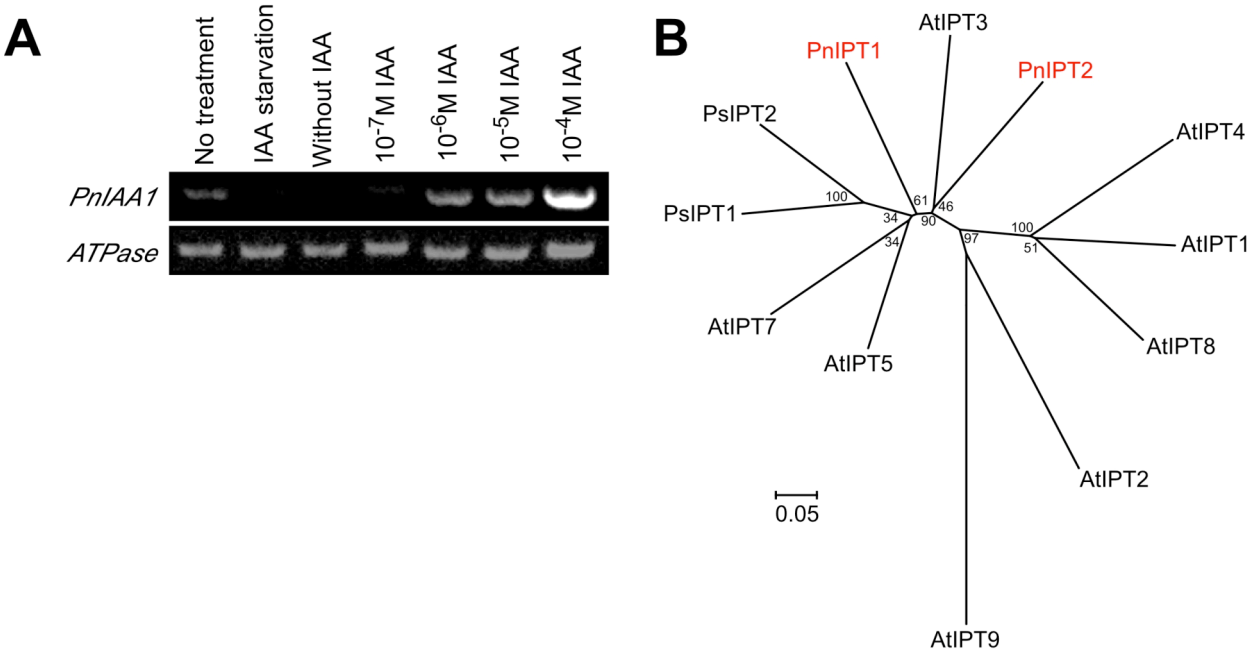


Fig. 3-4. Molecular identification of auxin and cytokinin reporter genes. (A) Auxin-responsiveness of the *PnIAA1* gene. One cm long stem segments were excised from three-week-old WT plants. Depletion of endogenous auxin and exogenous IAA treatment was accomplished according to Fujii *et al*. (2000). Total RNA was extracted from the incubated stem sections and subjected to semi-quantitative RT-PCR analysis. (B) Phylogenetic analysis of plant IPT family members. An unrooted phylogenetic tree was generated from multiple sequence alignments using ClustalW. The following sequences from the NCBI sequence database were used for the sequence alignment: AtIPT1 (AB061400), AtIPT2 (AB062609), AtIPT3 (AB061401), AtIPT4 (AB061402), AtIPT5 (AB061403), AtIPT6 (AB061404), AtIPT7 (AB061405), AtIPT8 (AB061406), AtIPT9 (AB062615), PnIPT1 (AB371300), PnIPT2 (AB371301), PsIPT1 (AB194606), PsIPT2 (AB194607). The phylogenetic tree was generated using the Neighbor Joining method and a distance matrix with correction for multiple substitutions. MEGA3 (http://www.megasoftware.net/mega.html) was used to draw the tree. Numbers at the branch forks represent the bootstrap values, which are expressed as a percentage of 1,000 bootstraps, and a confidence limit for the sequence groupings.

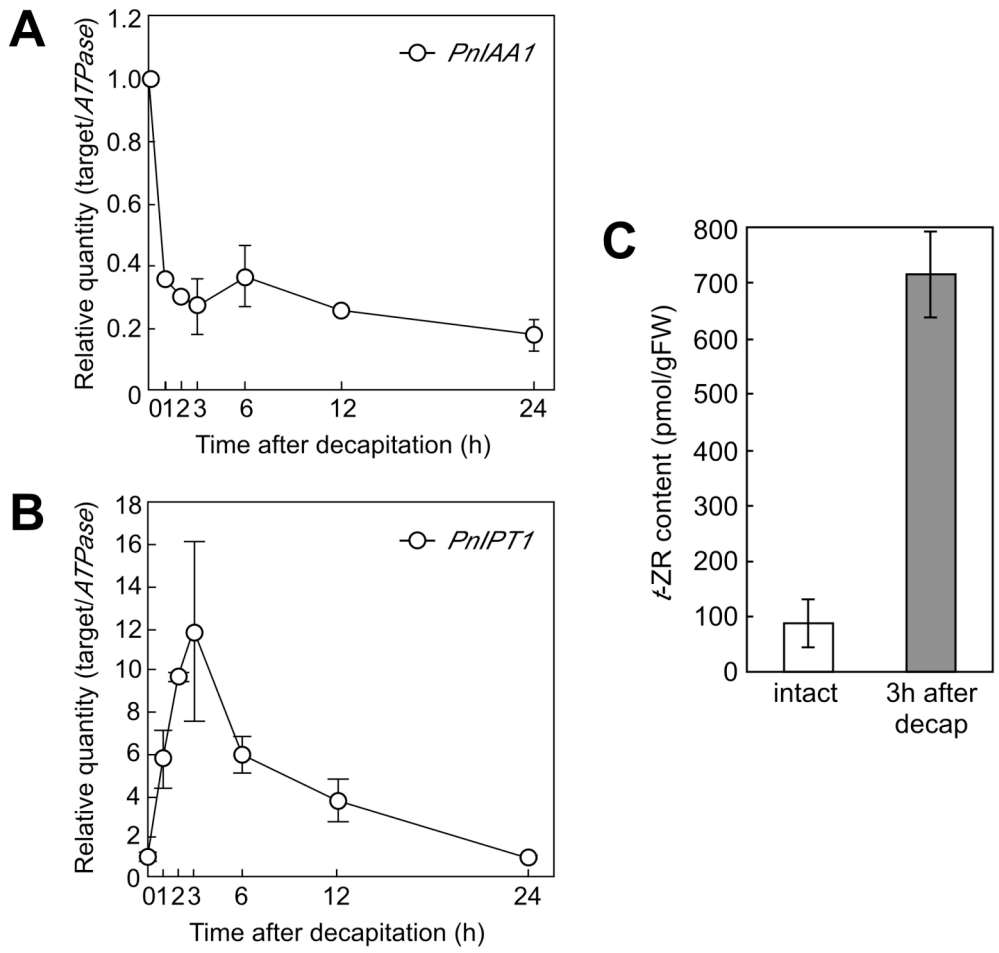


Fig. 3-5. Expression patterns of auxin and cytokinin marker genes and the level of endogenous *t*-ZR in the nodal stem after decapitation. Quantitative real-time PCR analysis of the expression of *PnIAA1* (A) and *PnIPT1* (B) was performed. The shoot apex was removed 1 cm above the first node, then the first nodal stems were collected at the indicated times. The expression levels of these genes were normalized to the expression of the mitochondrial F_1F_0 ATP synthase γ-subunit gene. Relative expression levels are shown. Samples that were obtained at 0 h were given a value of one. Data are the means ± SD. Experiments were performed in triplicate using independent RNA samples. (C) Endogenous *t*-ZR contents of the nodal stem after decapitation. The shoot apex was removed 1 cm above the first node, and then the first nodal stems were collected at the indicated times. The *t*-ZR contents were analyzed by ELISA as described in the Materials and Methods. Data are the means ± SE.

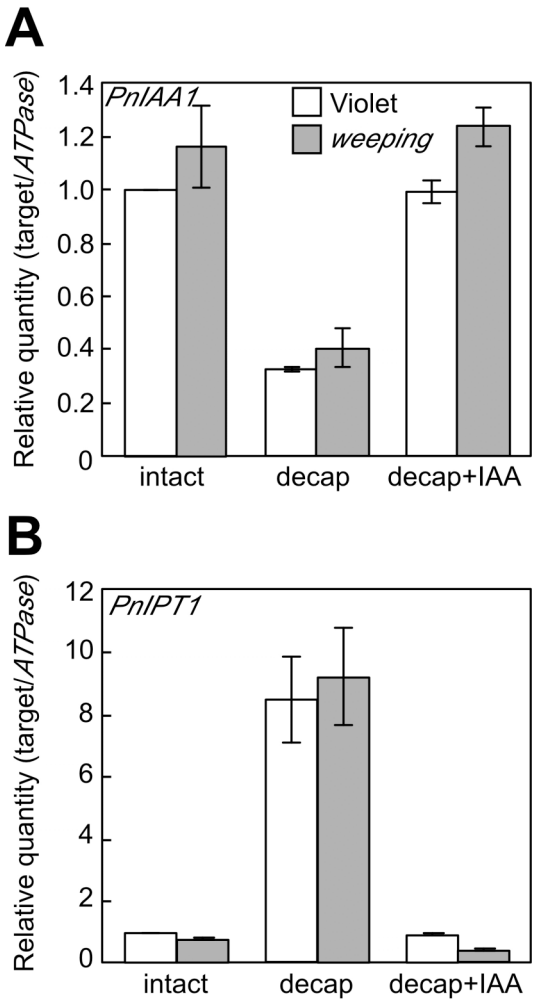


Fig. 3-6. Effects of IAA applied to the decapitated stump on *PnIAA1* and *PnIPT1* expressions in WT and *we* mutant plants. Quantitative real-time PCR analysis of the expression of *PnIAA1* (A) and *PnIPT1* (B) was performed. The shoot apex was removed 1 cm above the first node, and lanolin paste containing 0.1% IAA or without IAA was immediately applied to the stump. The first nodal stems were collected 3 h after treatment. The expression levels of these genes were normalized to the expression of the F_1F_0 ATP synthase γ-subunit gene. Relative expression levels are shown; that of Violet intact plants was set as one. Data are the means ± SD. Open column, Violet; shaded column, *weeping*.

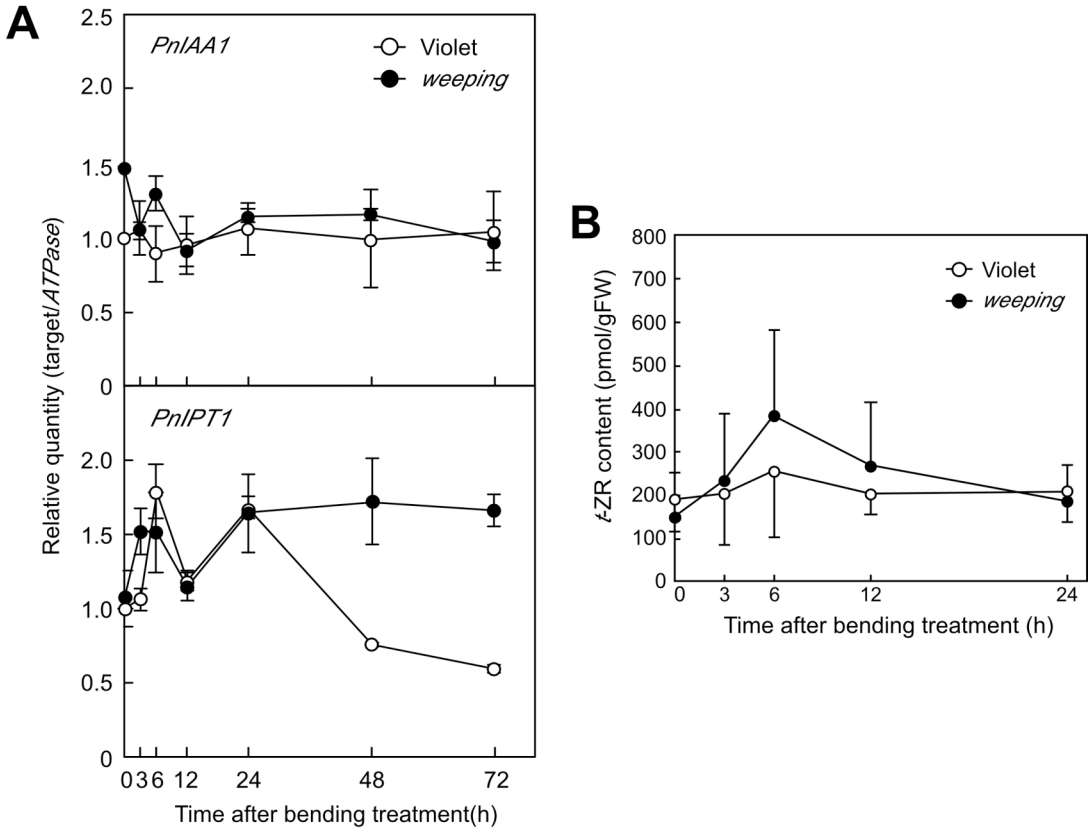


Fig. 3-7. The effects of shoot bending treatment on the expression of *PnIAA1* and *PnIPT1*, and endogenous *t*-ZR contents at the uppermost nodal stems of Violet and *weeping*. Bending treatment was performed, and then the uppermost nodes (the sixth node) were sampled at the indicated times after treatment. For non-treated plants, the node corresponding to the uppermost node (the sixth node) of the bent plants was sampled. (A) Quantitative real-time PCR analysis of the expression of *PnIAA1* (upper panel) and *PnIPT1* (lower panel) was performed. The expression levels of these genes were normalized to F_1F_0 ATP synthase γ-subunit gene expression levels. Relative expression levels are shown, and Violet plants at time zero (0 h) were given the value one. Data represent means ± SE. (B) Endogenous *t*-ZR contents of the nodal stem on the uppermost node during shoot bending treatment. The nodal stems on the uppermost node (the sixth node) were collected at the indicated times. The *t*-ZR contents were analyzed by ELISA as described in the Materials and Methods. Data represent means ± SE. Open circles, Violet; closed circles, *weeping*.

GENERAL DISCUSSION

The purpose of this study was to verify the involvement of the graviresponse in plant morphogenesis and to explore its molecular mechanism. I utilized agravitropic morning glory as a model plant, for it has distinctive features that allow us to approach plant gravimorphogenesis. I analyzed two distinct mutant lines of morning glory; *we* and *we2,* both of which have defects in shoot gravitropism. These mutants lacked the proper endodermal cells that are required for gravisensing in shoots. Interestingly, these mutants were absent from shoot circumnutation and winding growth. I found that one amino acid was inserted into the highly conserved GRAS domain in *we*-type PnSCR and that *we2*-type *PnSHR1* contained a single nucleotide transition that causes an amber mutation in the GRAS domain; these mutations caused abnormal endodermal differentiation. These results indicate that the aberrant phenotypes observed in *we* and *we2* are due to the mutations in *PnSCR* and *PnSHR1*, respectively. Various histological and molecular analyses using gravitropic mutants of *Arabidopsis* and morning glory have revealed that the endodermis-mediated graviresponse is indispensable for shoot circumnutation and the winding response. I have also succeeded in demonstrating that apical dominance is affected by the graviresponse. Previous studies have shown that basipetal translocation of auxin from the apical bud inhibits axillary bud growth, whereas cytokinin promotes axillary bud outgrowth. I therefore compared the roles of auxin and cytokinin in bending- and decapitation-induced axillary bud growth. The results implied that the mechanism underlying the gravity-regulated release of axillary buds from apical dominance differs from that of the decapitation-induced response.

1. *WEEPING* and *WEEPING2* were identified as regulatory genes for climbing behavior in plants

The Japanese morning glory (*Pharbitis nil* or *Ipomoea nil*) is a traditional horticultural plant in Japan. Many spontaneous mutants affecting the color and shape of the leaves and flowers have been isolated. The *weeping* morning glory “Shidare-asagao” was discovered in 1953 and has long been known to Japanese horticulturalists. To date, Japanese researchers have identified the genes that are responsible for several mutations in morning glory (Fukuda-Tanaka *et al*. 2000); however, only mutants that are defective in flower color or pigmentation have been characterized (Fukuda-Tanaka *et al*. 2000). The *WEEPING* and *WEEPING2* loci were the first examples of genes that are responsible for growth and morphology to be identified in the Japanese morning glory.

Morning glory is a typical climbing plant. Climbing habits include several distinct features such as twining and clambering with adhesive appendages. Morning glory is classified as a stem twining plant (Darwin 1876). Surprisingly, *weeping* morning glories are the first climbing plants to be reported that bear a mutation that affects the differentiation of the endodermis. Furthermore, the *WEEPING* locus is the first example of a gene that is responsible for defects in climbing plants. The stems of twining plants grow around a cylindrical support in a corkscrew shape that is geometrically regular, so that the stem forms a helical tube of tissue with uniform curvature and torsion (Scher *et al*. 2001). The vine shoots of the two *weeping* mutants completely lack the ability to wind around a support; therefore, *PnSCR* and *PnSHR1* might also be required for the winding

response in morning glory.

2. PnSCR and PnSHR1 regulate the pleiotropic phenotype in morning glory

It appears that *weeping* mutants show pleiotropic phenotypes due to the mutation of a single gene. How does this arise? One simple interpretation is that these phenotypes are directly regulated by the genes responsible for them, namely *PnSCR* and *PnSHR1*. These genes have been well characterized in *Arabidopsis*. SCR and SHR are key regulators of shoot and root radial patterning (Di Laurenzio *et al*. 1996, Fukaki *et al*. 1998, Helariutta *et al.* 2000). The *Arabidopsis scr* and *shr* mutants have an abnormally differentiated endodermis and lack the gravitropic response in their shoots (Fukaki *et al*. 1998). SHR is expressed in the stele, and then transported into the adjacent cell layer where it regulates SCR transcription and endodermal specification (Nakajima *et al*. 2001). SCR is predominantly expressed in the endodermis, the cortex/endodermis initial (CEI) cell, and the quiescent center (QC), and is required for asymmetric cell division that gives rise to the cortex and endodermis (Di Laurenzio *et al*. 1996, Gallagher *et al*. 2004). Thus, SHR and SCR cooperatively regulate endodermal development.

Both SHR and SCR belong to the GRAS transcription factor family, which regulate diverse aspects of plant growth and development (Tian *et al*. 2004). All GRAS proteins share a conserved C-terminal GRAS domain, but their N termini are more divergent. The GRAS domain contains several conserved motifs, including two-leucine-heptad repeats (LHR I, II), VHIID, PFYRE, and SAW (Tian *et al*. 2004). The LHR I-VHIID-LHR II region

might function as a DNA-binding domain, modestly analogous to the basic leucine zipper motif that is known to play an important role in various aspects of plant growth and development (Jakoby *et al*. 2002). Recently, it was shown that SHR directly activates the transcription of *SCR* and other several genes whose function remains to be determined (Levesque *et al*. 2006). In this report, the authors showed that SHR directly binds to the promoter of four genes, *MGP*, *NUC*, *TRI* and *SCR*, although whether the basic leucine zipper motif in SHR binds to those promoters was unknown. MGP and NUC appeared to belong to the C2H2 zinc finger transcription factor family. TRI might regulate tropane alkaloid synthesis. Thus, the SHR/SCR-dependent transcriptional system may include many downstream factors in *Arabidopsis*.

It is possible that PnSCR and PnSHR1 also activate similar target genes in morning glory. The genes that are downstream of *PnSCR*/*PnSHR* might also be involved in endodermal development. Alternatively, it is possible that these genes not only facilitate endodermal differentiation but also modulate events that are upstream of gravimorphogenesis, and do not participate in the graviresponse. Indeed, it is known that inflorescence stems from the gravitropic *Arabidopsis* mutant*, sgr4/zig*, which has an endodermis with abnormal amyloplast sedimentation and vacuolar morphology, exhibits “zigzag” growth under conventional conditions (Kato *et al*. 2002a). *SGR4/ZIG* encodes a v-SNARE-like protein, AtVTI11, which may act on vacuolar biogenesis in whole plant organs. Zigzag growth appears to be caused by abnormal vacuolar biogenesis due to the *sgr4/zig* mutation; this is not related to the graviresponse.

With respect to the *weeping* mutants; however the latter hypothesis is unlikely,

because the two *weeping* morning glories show normal apical dominance when they are grown under conventional conditions (see Chapter 3). Furthermore, the two *weeping* mutants respond to decapitation (see Chapter 3). The abnormal elongation of the axillary buds in *weeping* plants is only seen when their stems are bent down (see Chapter 3). This bending-induced response is apparently controlled by the endodermis-mediated graviresponse, but is not affected by graviresponse-independent factors. Consequently, it appears that WE/PnSCR and WE2/PnSHR1 generate the gravisensing endodermis which induces the graviresponse, and thereby pleiotropic phenotypes such as circumnutation and the release of axillary buds from apical dominance occur.

3. A possible role of lateral auxin transport in the pleiotropic phenotypes of morning glory

The answer to the question of how a pleiotropic phenotype is generated by the mutation of a single gene is hidden in the gravisensing endodermal cells that contain sediment amyloplasts. During gravitropism, the movement (sedimentation) of starch-filled plastids called amyloplasts along the gravity vector within gravisensing cells (statocytes) is most likely to be a trigger of subsequent intracellular signaling (Blancaflor and Masson 2003, see the general introduction). In shoots, sedimenting amyloplasts have been observed in the endodermal cells of hypocotyls and the stems of dicotyledonous plants. When amyloplasts within statocytes sediment along the gravity vector this mechanical stimulus is converted into a biological signal. In roots, this signal directs the localization of auxin transport facilitators in statocytes, and thereby auxin is

asymmetrically transported to the response site where it causes a curvature-response that allows the organ tip to resume growth at a predefined set angle from the gravity vector (Masson *et al*. 2002). In shoots, endodermis-mediated gravisensing may also lead to lateral auxin transport, although its mechanism remains unclear (Friml *et al*. 2002). It is therefore possible that the lack of a normal endodermis in *weeping* mutants abolishes lateral auxin transport in response to gravistimutation. In this study, I examined lateral auxin transport using ^{3}H-labeled IAA in WT and *we* plants (Supplementary Table 1). In WT plants, when the hypocotyl sections were maintained in a horizontal position, ^{3}H in the longitudinally halved lower sections increased compared with that in the upper sections. In contrast, placing the hypocotyl sections in a horizontal position did not cause this ^{3}H asymmetry in *we* plants, indicating that the lateral transport activity of auxin is significantly reduced in *we* plants. This result suggests that gravity-related lateral translocation of auxin is impaired in *we* shoots.

It has been shown that the polar localization of auxin transport facilitators such as PIN-FORMED (PIN) proteins in statocytes plays a role in gravity-regulated auxin transport (Friml *et al*. 2002, see the general introduction). It is possible that PIN proteins are abnormally expressed or localized in the shoots of *weeping* mutants, as *we* plants have defects in the lateral translocation of auxin. I therefore investigated the expression of *PIN* genes in WT and *weeping* morning glory plants (Supplementary Fig. 1). I first isolated partial cDNAs for four distinct *PIN* genes using degenerated-PCR from morning glory. Preliminary RT-PCR analysis revealed that all four *PIN* genes are expressed in WT morning glory stems. I then carried out real-time PCR analysis in order to analyze

the expression of the *PIN* genes. The results showed that the mRNA level of *PnPIN1* was reduced in both *we* and *we2* mutants, and the mRNA level of *PnPIN4* was reduced in *we* but not in *we2* (Supplementary Fig. 1). This suggests that these two *PIN* genes are expressed in endodermal cells because *weeping* mutants lack that cell layer. Furthermore, these results imply that the impaired activity of lateral auxin translocation in *we* plants is due to the reduced expression of *PnPIN1* and/or *PnPIN4*, although whether these candidates act as auxin facilitators remains unknown.

Circumnutation and apical dominance are thought to be auxin-related phenomena (see the general introduction). The two-oscillator model is widely accepted to explain the mechanism regulating circumnutation. The internal oscillator has been modeled as a growth wave traveling around the elongating organs that could be coupled with the oscillation of growth substances such as auxin (Johnsson *et al*. 1999). Endodermis-mediated gravisensing is thought to lead to the lateral translocation of auxin, which causes sequential asymmetric growth of elongating stems, and thereafter nutational movements occur. Endodermis-less *weeping* plants may therefore display impaired circumnutation because auxin asymmetry does not occur in their stem.

Auxin is thought to have a major role in the control of apical dominance. Namely, basipetal translocation of auxin is inhibitory to the growth of axillary buds. In contrast, my study suggests that the gravity-regulated release of axillary buds from apical dominance is not related to basipetal auxin transport (see Chapter 3). Alternatively, bending-induced release of axillary buds from apical dominance could be related to lateral auxin transport that is modified by gravistimulation. In order to verify this possibility, however, the

detailed distribution of auxin in bending shoots needs to be examined.

4. What is the intimate factor governing gravimorphogenesis in plants?

This study revealed that circumnutation, winding, and apical dominance depend on the endodermis-mediated graviresponse. *WEEPING* and *WEEPING2* encode PnSCR and PnSHR1, respectively; these proteins are indispensable for the normal development of endodermal cells. The endodermis is considered to be the gravisensing tissue that is necessary for shoot gravitropism; therefore, the gravity-dependent morphogenesis mechanism shares, at least in part, an early step of the graviresponse cascade with that of gravitropism.

What is a crucial substance or molecule governing gravity-related phenomena such as circumnutation, winding and apical dominance? The answer may be hidden in the pathway between graviperception and the asymmetrical redistribution of auxin. These factor(s) could also be shared by gravitropism and other gravity-influenced morphogenesis. It is also possible that mechanisms that are innate to specific plant species exist. For example, the direction of shoot circumnutation differs among plant species. In brief, shoots from morning glory display left-handed rotation whereas those from *Arabidopsis* altenately rotate (Schuster and Engelmann 1997). In addition, winding growth is seen in a limited number of plant species, suggesting that they have specialized growth mechanics that connect with the graviresponse.

5. Conclusion

In this study, I have succeeded in identifying the genes responsible for the *weeping*

phenotype in morning glory mutants (*we* and *we2*). I used these two mutant lines of morning glory to verify the involvement of the graviresponse in gravimorphogenesis including circumnutation, winding and apical dominance (Supplementary Fig. 2).

It is now important to discover substances or molecules that are involved in regulating gravity-dependent morphogenesis in plants. One way to approach this issue is to analyze an agravitropic morning glory that has normal endodermal cells and sediment amyloplasts. Such a mutant would be useful for identifying pathways downstream of graviperception. Another approach would be to perform a comparative analysis between WT and *weeping* mutants by the large scale scanning of gene or protein expression, such as the use of cDNA arrays or proteomics. Morning glory has distinctive features such as winding; therefore, novel findings finding could be expected which had not been previously obtained from the analysis of conventional model plants. Once the candidate genes had been identified, their function in gravity-dependent morphogenesis could be demonstrated by the molecular genetic analysis of morning glory plants in which those specific genes had been manipulated. The results from a comprehensive analysis such as this would then need to be integrated with our current knowledge in order to fully understand gravity-regulated mechanisms.

Winding and apical dominance are important and beneficial traits from an agricultural or horticultural point of view. They strongly affect growth and yield of crops as well as the ornamental value of horticultural plants. Understanding the gravity-regulated mechanisms of these phenomena will allow us to develop new technology that could improve the cultivation of agricultural and horticultural plants. This

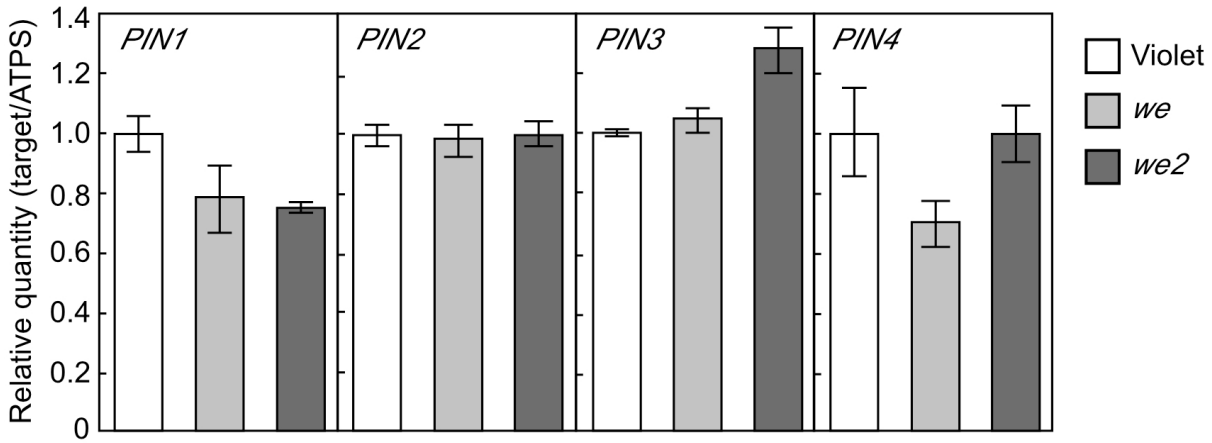
study has emphasized the use of agravitropic morning glory as a model plant for the study of gravity-regulated morphogenesis and its molecular mechanism.

Supplementary Table 1.

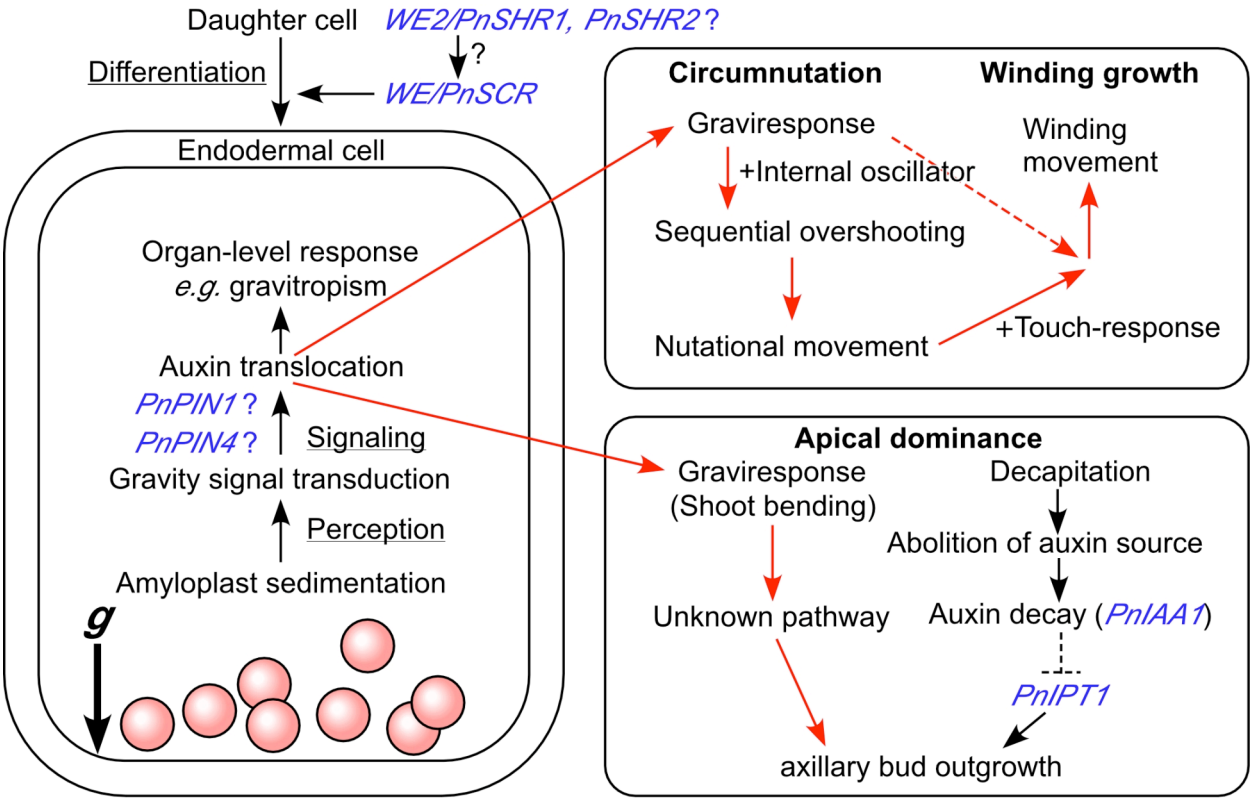
Lateral transport activity of IAA in Violet (WT) and *we* hypocotyls

	Upright-left (cpm)	Upright-right	Horizontal-upper	Horizontal-lower
Violet	434.3 ± 29.8	436.8 ± 19.1	450.1 ± 28.8	587.3 ± 34.2
we	455.4 ± 19.2	476.9 ± 42.7	391.1 ± 14.4	427.5 ± 28

This experiment was performed according to the method used to analyze the basipetal transport activity of IAA described in Chapter 3. For the measurement of IAA lateral transport, vials containing hypocotyl sections were kept in either an upright or horizontal position for 5 h. After incubation, a 1-cm-long middle portion of each section was excised and halved transversely. ^{3}H levels in the halved middle portion (left-right or upper-lower) were estimated using 5 ml of liquid scintillation cocktail and a liquid scintillation counter (LS6500; Beckman). At least 10 hypocotyl sections were counted for each sample. Data are the means ± SE.



Supplementary Fig. 1. Quantitative RT-PCR analysis of the expression of *PIN-FORMED* (*PIN)* genes in the stems of morning glory. Total RNA was extracted from the stems of 14-day-old morning glory plants grown in a greenhouse. First-strand cDNA was synthesized as described in chapter 1. Real-time RT-PCR was performed using a MyiQ single-color real-time PCR detection system according to the manufacturer's instructions. Calibration was performed using a plasmid-based cDNA template. Target gene expression was normalized to the expression of the mitochondrial F_1F_0 ATP synthase γ-subunit, and relative expression levels are shown. Data represents the averages and standard deviation of triplicate experiments. The PCR conditions were as follows: 120 seconds (s) at 95°C; 45 cycles of 10 s at 95°C, 20 s at 60°C (for *PnPINs*) or 20 s at 55°C (for *ATPS*), 30 s at 72°C followed by melting curve analysis. Primers used for the amplification of each gene are shown in Table 1-1 (see Chapter 1).



Supplementary Fig. 2. Schematic representation of the graviresponse pathway regulating oscillatory movements and apical dominance. WE/PnSCR and WE2/PnSHR1 cooperatively play roles in endodermal development. In response to gravity, sedimenting amyloplasts within the endodermis provide a mechanical stimulus that is converted into a biological signal and determines the direction of auxin transport. PnPIN1 and/or PnPIN4 may be involved in this process. Asymmetrically distributed auxin causes various responses in organs. The auxin gradient across gravistimulated tissues causes differential growth, and thereby induces a gravitropic response. In circumnutation, gravity-dependent auxin translocation might affect the rhythmic wave of an internal oscillator such as auxin or calcium, which might induce nutational movement. In contrast, the gravity-regulated release of axillary buds from apical dominance can be explained by a mechanism that is distinct from that of decapitation-induced release; basipetal auxin transport and cytokinin biosynthesis are responsible for the latter but not for the former. Thus, graviperception mediated by endodermal cells brings about pleiotropic phenomena.

SUMMARY OF THIS STUDY

Introduction

During the course of evolution, plants have acquired the ability to sense gravity and have thereby adapted to the terrestrial environment by controlling their morphological features (Masson *et al*. 2002). This gravity-dependent morphogenesis in plants is called gravimorphogenesis (Takahashi 1997). One typical example is gravitropism that enables plants to orient their shoots upward and roots downward (Masson *et al*. 2002). A number of studies have paid much attention to gravitropism, providing us with many clues to understand its mechanistic aspects (Blancaflor & Masson 2003). The endodermis and columella cells are thought to be gravisensing cells in shoots and roots, respectively (Blancaflor & Masson 2003). Numerous studies have suggested that sedimentation of amyloplasts in gravisensing cells is required for gravisensing (Morita & Tasaka 2004). In addition to the studies of gravitropism, it has been suggested that gravity also affects various aspects of plant growth such as circumnutation, winding and apical dominance (Darwin & Darwin 1881, Cline 1991). In contrast to gravitropism; however, mechanisms of the latter type of gravimorphogenesis remain unclear, and the relationship between the graviresponse and morphogenesis still needs to be verified. Characterization of gravitropic mutants is a helpful approach because phenotypes that genetically link the graviresponse can be identified and analyzed directly. I utilized gravitropic mutants of the Japanese morning glory (*Pharbitis nil* or *Ipomoea nil*) to study of the mechanisms regulating gravity-dependent circumnutation/winding and the gravity-stimulated

outgrowth of axillary buds. In this study I characterized two distinct agravitropic mutants of morning glory, *weeping* (*we*) and *weeping2* (*we2*), identified the genes responsible for their aberrant phenotypes and demonstrated a genetic link between the graviresponse and circumnutation, winding and apical dominance, using *weeping* mutants of morning glory together with several gravitropic mutants of *Arabidopsis thaliana*.

Phenotype of the we mutant and molecular identification of its locus

The stem of WT morning glory displays gravitropism, whereas the stems of both *we* and *we2* mutants do not display this phenomenon. The *we* mutant completely lacks a gravitropic response in both the stem and hypocotyl, whereas its roots are gravitropically normal (Hatakeda *et al.* 2003). It was previously demonstrated that *we* lacks a normal endodermal cell layer containing sedimentable amyloplasts (Hatakeda *et al.* 2003). This phenotype appeared to be similar to that of the *Arabidopsis scarecrow* (*scr*) mutant. I therefore examined whether the abnormal phenotypes of *we* were due to changes in gene expression or loss-of-function of the morning glory *SCR* (*PnSCR*). I found that one amino acid was inserted into the highly conserved GRAS domain in *we*-type PnSCR. To confirm whether this mutation was responsible for the *we* phenotype, I introduced either the mutant- or WT-*PnSCR* into the *Arabidopsis scr* mutant for complementation tests. *PnSCR* from the WT, but not from *we*, rescued endodermal development and shoot gravitropism in the *scr* mutants. These results show that the defects in endodermal development and lack of shoot gravitropism in *we* are attributable to a loss of PnSCR function (Kitazawa *et al*. 2005).

Phenotypic and genetic characterization of we2 mutants

Shoot gravitropism is not evident in *we2* mutants. To characterize the gravitropic response of *we2* plants in more detail, I carried out a time-course analysis of the gravitropic responses of *we2* hypocotyls and roots. In contrast to *we*, hypocotyls of *we2* exhibited a reduced gravitropic curvature, while roots showed normal gravitropism. Histological analysis showed that *we2* mutants formed abnormal endodermal cells, similar to those observed in the *we* mutant. In *Arabidopsis*, the cooperative action of *SCR* and *SHORT-ROOT* (*SHR*) plays a key role in endodermal development in the shoots and roots (Fukaki *et al*. 1998). Since *we2* and *we* occupy separate genomic loci, I examined whether the abnormal phenotype of *we2* mutants was caused by a mutation of the morning glory homolog of *SHR*. I found that the *we2*-type *SHORT-ROOT* homolog (*PnSHR1*) contained a single nucleotide transition that could cause an amber mutation in the GRAS domain. Also, this mutation was genetically linked to the agravitropism of *we2*. These results indicate that both abnormal endodermal development and gravitropism in *we2* are due to the expression of a truncated *PnSHR1* gene product (Kitazawa *et al*. 2008). In addition, *PnSCR* expression was remarkably reduced in the stems and hypocotyls of *we2*, which suggests that *PnSHR1* functions upstream of *PnSCR* during endodermal development in morning glory.

Investigation of the relationship between the graviresponse and circumnutation

Plant organs undergo oscillatory movements termed circumnutation (Johnsson

1997). It is believed that circumnutation provides the motive power for the winding response of some climbing plants (Darwin & Darwin 1881). The gravity-dependency of these oscillatory movements has long been a controversial matter (Johnsson 1997). To date, it remains obscure whether the graviresponse is indispensable for oscillatory movements. To obtain conclusive evidence for the involvement of the graviresponse in circumnuation, I analyzed the oscillatory movements of multiple gravitropic mutants of the morning glory and *Arabidopsis*. My results revealed that both circumnutation and the winding response of the vines was impaired in *we2* mutants, as reported for *we* mutant (Hatakeda *et al*. 2003). In addition, *we* hypocotyls did not show an explicit circumnutation, but in contrast, *we2* hypocotyls displayed a slight nutational movement. These results strongly suggest that oscillatory movements are linked to the ability to generate a gravitropic response. Introducing WT *PnSCR* to *scr Arabidopsis* restored not only endodermal development but also shoot circumnutation. Moreover, shoot circumnutation in *sgr2* and *sgr4/zig Arabidopsis*, which have endodermal cell layers with abnormal amyloplast sedimentation (Kato *et al*. 2002a), was severely reduced. These results indicate that a functioning endodermis is required for shoot circumnutation and winding movements in the morning glory plants. That is, shoot circumnutation depends on the endodermis-mediated graviresponse.

Investigation of the relationship between the graviresponse and apical dominance

In many plant species, the apical shoot grows predominantly and represses axillary bud growth. This phenomenon is called apical dominance (Cline 1991). Apical

dominance is best demonstrated via shoot tip removal, also known as decapitation, which releases axillary buds from apical dominance and stimulates their outgrowth (Cline 1991). When the upper part of the main shoot of morning glory is bent down, the axillary bud situated on the uppermost node of the bending region is released from apical dominance and elongates (Prasad & Cline 1987). This release of axillary buds from apical dominance is prevented by clinorotation of the bent plants, suggesting the involvement of the graviresponse in apical dominance (Prasad & Cline 1987). To date, there has been no direct evidence for a relationship between the graviresponse and apical dominance. In order to obtain genetic evidence that the graviresponse regulates apical dominance, I utilized the *weeping* mutants. Bending the main shoots of either *we* or *we2* plants did not induce elongation of their axillary buds. Linkage analysis using F_2 generations crossed with WT morning glory revealed that this aberration was linked to the agravitropism phenotype of *weeping* mutants. This implies that bending-induced release from apical dominance requires the graviresponse. It has been shown that basipetal translocation of auxin from the apical bud inhibits axillary bud growth, whereas cytokinin promotes axillary bud outgrowth (Shimizu-Sato & Mori 2001). I therefore compared the roles of auxin and cytokinin in bending- and decapitation-induced axillary bud growth. In the WT morning glory, decapitation increased cytokinin levels and reduced the auxin response. In contrast, shoot bending did not cause significant changes in either cytokinin level or auxin response. These results imply that the mechanisms underlying the gravity- and decapitation-regulated release from apical dominance are distinct and unique.

Conclusive remarks

In this study, I have identified *WE*/*PnSCR* and *WE2*/*PnSHR1* as the genes responsible for agravitropic growth in the *we* and *we2* mutants of the morning glory, respectively. Using these two distinct morning glory mutants, I succeeded in demonstrating the involvement of the graviresponse in circumnutation, the winding response and apical dominance. This work demonstrates that *weeping* mutants are useful as a model system for exploring the mechanisms of gravimorphogenesis in plants. The identification of factors that function downstream of PnSCR and PnSHR1 in morning glory will provide further information as to how the graviresponse regulates plant morphogenesis such as circumnutation and apical dominance.

REFERENCES

Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Winiewska, J., Moulinier-Anzola, J.C., Sieberer, T., Friml, J. and Luschnig, C. (2006) Intracellular trafficking and proteolysis of the *Arabidopsis* auxin-efflux facilitator PIN2 are involved in root gravitropism. *Nat. Cell Biol*. 8: 249–256.

Abdel-Rahman, A.M. and Cline, M.G. (1989) Timing of growth inhibition following shoot inversion in *Pharbitis nil*. *Plant Physiol*. 91: 464–465.

Abe, K., Takahashi, H. and Suge, H. (1998) Gravimorphism in rice and barley: promotion of leaf elongation by vertical inversion in agravitropically growing plants. *J. Plant Res*. 111:523–530.

Adamchuk, N.I. (1998) Ultrastructural and functional changes of photosynthetic apparatus of *Arabidopsis thaliana* (L.) heynh induced by clinorotation. *Adv. Space Res*. 21: 1131–1134.

Blakeslee, J.J., Peer, W.A. and Murphy, A.S. (2005) Auxin transport. *Curr. Opin. Plant Biol*. 8: 494–500.

Blancaflor, E.B., Fasano, J.M. and Gilroy, S. (1998) Mapping the functional roles of root cap cells in response of *Arabidopsis* primary roots to gravity. *Plant Physiol*. 116: 213–222.

Blancaflor, E.B. and Masson, P.H. (2003) Plant gravitropism. Unraveling the ups and downs of a complex process. *Plant Physiol*. 133: 1677–1690.

Booker, J., Chatfield, S. and Leyser, O. (2003) Auxin acts in xylem-associated or medullary cells to mediate apical dominance. *Plant Cell* 15: 494-507.

Boonsirichai, K., Sedbrook, J.C., Chen, R., Gilroy, S. and Masson, P.H. (2003) ALTERED RESPONSE TO GRAVITY is a peripheral membrane protein that modulates gravity-induced cytoplasmic alkalinization and lateral auxin transport in plant statocytes. *Plant Cell* 15: 2612–2625.

Brown, A.H. and Chapman, D.K. (1984) Circumnutation observed without a significant gravitational force in spaceflight. *Science* 225: 230–232.

Brown, A.H., Chapman, D.K., Lewis, R.F. and Venditti, A.L. (1990) Circumnutations of sunflower hypocotyls in satellite orbit. *Plant Physiol*. 94: 233–238.

Brown, B.T., Foster, C., Phillips, J.N. and Rattigann, B.M. (1979) The indirect role of 2,4-D in the maintenance of apical dominance in decapitated sunflower seedlings (*Helianthus annuus* L.). *Planta* 146: 475–480.

Catterou, M., Dubois, F., Smets, R., Vaniet, S., Kichey, T., Van Onckelen, H., Sangwan-Norreel, B.S. and Sangwan, R.S. (2002) *hoc*: An *Arabidopsis* mutant overproducing cytokinins and expressing high in vitro organogenic capacity. *Plant J.* 30: 273–287.

Cline, M.G. (1983) Apical dominance in *Pharbitis nil*: effects induced by inverting the apex of the main shoot. *Ann.Bot*. 52: 217–227.

Cline, M.G. and Riley, L. (1984) The presentation time for shoot inversion release of apical dominance in *Pharbitis nil*. *Ann. Bot*. 53: 897–900.

Cline, M.G. (1991) Apical dominance. *Bot. Rev*. 57: 318–358.

Cline, M., Wessel, T. and Iwamura, H. (1997) Cytokinin/auxin control of apical dominance in *Ipomoea nil*. *Plant Cell Physiol*. 37: 659–667.

Clough, S. and Bent, A. (1998) Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J*. 16, 735–743.

Cui, H., Levesque, M.P., Vernoux, T., Jung, J.W., Paquette, A.J., Gallagher, K.L., Wang, J.Y., Blilou, I., Scheres, B. and Benfey, P.N. (2007) An evolutionarily conserved mechanism delimiting SHR movement defines a single layer of endodermis in plants. *Science* 316: 421–425.

Darwin, C. (1876) *Movements and habits of climbing plants*. John Murray, London.

Darwin, C. and Darwin, F. (1881) *The Power of Movement in Plants*. John Murray, London.

Di Laurenzio, L., Wysocka-Diller, J., Malamy, J.E., Pysh, L., Helariutta, Y., Freshour, G., Hahn, M. G., Feldmann, K. A. and Benfey, P.N. (1996) The *SCARECROW* gene regulates an asymmetric cell division that is essential for generating the radial organization of the *Arabidopsi*s root. *Cell* 86: 423–433.

Fasano, J.M., Swanson, S.J., Blancaflor, E.B., Dowd, P.E., Kao, T.H. and Gilroy, S. (2001) Changes in root cap pH are required for the gravity response of the *Arabidopsis* root. *Plant Cell* 13: 907–921.

Friml, J., Wisniewska, J., Benkova, E., Mendgen, K. and Palme, K. (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. *Nature* 415: 806–809.

Fujihira , K., Kurata, T., Watahiki, M., Karahara, I. and Yamamoto, K. (2000) An agravitropic mutant of *Arabidopsis*, *endodermal-amyloplast less 1*, that lacks amyloplasts in hypocotyl endodermal cell layer. *Plant Cell Physiol*. 41: 1193–1199.

Fujii, N., Kamada, M., Yamasaki, S. and Takahashi, H. (2000) Differential accumulation of *Aux/IAA* mRNA during seedling development and gravity response in cucumber (*Cucumis sativus* L.). *Plant Mol. Biol*. 42: 731–740.

Fukaki, H., Wysocka-Diller, J., Kato, T., Fujisawa, H., Benfey, P.N. and Tasaka, M. (1998) Genetic evidence that the endodermis is essential for shoot gravitropism in *Arabidopsis thaliana*. *Plant J.* 14: 425–430.

Fukuda-Tanaka, S., Inagaki, Y., Yamaguchi, T., Saito, N. and Iida, S. (2000) Colour-enhancing protein in blue petals. *Nature* 407: 581 (brief communications).

Gallagher, K.L., Paquette, A.J., Nakajima, K. and Benfey, P.N. (2004) Mechanisms regulating SHORT-ROOT intercellular movement. *Curr. Biol.* 14: 1847–1851.

Gocal, G.F., Pharis, R.P., Yeung, E.C. and Pearce, D. (1991) Changes after decapitation in concentrations of indole-3-acetic acid and abscisic acid in the larger axillary bud of *Phaseolus vulgaris* L. cv Tender Green. *Plant Physiol*. 95: 344-350.

Golovko, A., Sitbon, F., Tillberg, E. and Nicander, B. (2002) Identification of a tRNA isopentenyltransferase gene from *Arabidopsis thaliana*. *Plant Mol. Biol*. 49: 161–169.

Guilfoyle, T.J. and Hagen, G. (2007) Auxin response factors. *Curr. Opin. Plant Biol.* 10: 453–460.

Hatakeda, Y., Kamada, M., Goto, N., Fukaki, H., Tasaka, M., Suge, H. and Takahashi, H.

(2003) Gravitropic response plays an important role in the nutational movements of the shoot of *Pharbitis nil* and *Arabidopsis thaliana*. *Physiol. Plant*. 118: 464–473.

Helariutta, Y., Fukaki, H., Wysocka-Diller, J., Nakajima, K., Jung, J., Sena, G., Hauser, M. and Benfey, P.N. (2000) The *SHORT-ROOT* gene controls radial patterning of the *Arabidopsis* root through radial signaling. *Cell* 101: 555–567.

Harrison, B.R. and Masson, P.H. (2007) ARL2, ARG1 and PIN3 define a gravity signal transduction pathway in root statocytes. *Plant J.* (doi: 10.1111/j. 1365-313X.2007.03351.x)

Hoshino, A., Johzuka-Hisatomi, Y., Iida, S. (2001) Gene duplication and mobile genetic elements in the morning glories. *Gene* 265: 1–10.

Hoson, T., Kamisaka, S., Masuda, Y. and Yamashita, M. (1992) Changes in plant growth processes under microgravity conditions simulated by a three-dimensional clinostat. *Bot. Mag.* 105: 53–70.

Hoson, T., Nishitani, K., Miyamoto, K., Ueda, J., Kamisaka, S., Yamamoto, R. and Masuda, Y. (1996) Effects of hypergravity on growth and cell wall properties of cress hypocotyls. *J. Exp. Bot*. 47: 513–517.

Hoson, T., Saiki, M., Kamisaka, S. and Yamashita, M. (2001) Automorphogenesis and gravitropism of plant seedlings grown under microgravity conditions. *Adv. Space Res*. 27: 933–940.

Hoson, T., Soga, K., Mori, R., Saiki, M., Nakamura, Y., Wakabayashi, K. and Kamisaka, S. (2002) Stimulation of elongation growth and cell wall loosening in rice coleoptiles under microgravity conditions in space. *Plant Cell Physiol*. 43: 1067–1071.

Hoson, T. and Soga, K. (2003) New aspects of gravity response in plant cells. *Int. Rev. Cytol*. 229: 209–244.

Hou, G., Mohamalawari, D.R. and Blancaflor, E.B. (2003) Enhanced gravitropism of roots with a disrupted cap actin cytoskeleton. *Plant Physiol.* 131: 1360-1373.

Hou, G., Kramer, V.L., Wang, Y.S., Chen, R., Perbal, G., Gilroy, S. and Blancaflor, E.B. (2004) The promotion of gravitropism in *Arabidopsis* roots upon actin disruption is coupled with the extended alkalinization of the columella cytoplasm and a persistent lateral auxin gradient. *Plant J*. 39, 113–125.

Israelsson, D. and Johnsson, A. (1967) A theory for circumnutation in *Helianthus annuus*. *Physiol. Plant*. 20: 957–976.

Ito, A., Hayama, H. and Yoshida, H. (2001) The effect of shoot-bending on the amount of diffusible indole-3-acetic acid and its transport in shoots of Japanese pear. *Plant Growth Regul*. 34: 151–158.

Jaffe, M.J., Takahashi, H. and Biro, R.L. (1985) A pea mutant for the study of hydrotropism in roots. *Science* 230: 445–447.

Jakoby, M., Weisshaar, B., Dröge-Laser, W., Vicente-Carbajosa, J., Tiedemann, J., Kroj, T. and Parcy, F. (2002) bZIP transcription factors in *Arabidopsis*. *Trends Plant Sci*. 7: 106–111.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J*. 6: 3901–3907.

Johnsson, A. and Heathcote, D. (1973) Experimental evidence and models on

circumnutation. *Zeitschr. f. Pflanzenphysiol*. 70: 371–405.

Johnsson, A. (1977) Plant experiments: circumnutations under free-fall conditions in space. *Proc. R. Soc. Lond. B*. 199: 505–512.

Johnsson, A. (1979) Circumnutation. In: Haupt, W., Feinleib, M.E. (eds) Encyclopedia of Plant Physiology Vol. 7. Physiology of Movements. (Springer-Verlag, Berlin), pp. 627–646.

Johnsson, A. (1997) Circumnutations: results from recent experiments on Earth and in space. *Planta* 203: S147–S158.

Johnsson, A., Jansen, C., Engelmann, W. and Schuster, J. (1999) Circumnutations without gravity: a two-oscillator model. *J. Grav. Physiol*. 6: 9–12.

Kakimoto, T. (2003) Biosynthesis of cytokinins. *J. Plant Res*. 116: 233–239.

Kamada, M., Fujii, N., Aizawa, S., Kamigaichi, S., Mukai, C., Shimazu, T. and Takahashi, H. (2000) Control of gravimorphogenesis by auxin: accumulation pattern of *CS-IAA1* mRNA in cucumber seedlings grown in space and on the ground. *Planta* 211: 493–501.

Kamada, M., Yamasaki, S., Fujii, N., Higashitani, A. and Takahashi, H. (2003) Gravity-induced modification of auxin transport and distribution for peg formation in cucumber seedlings: possible roles for *CS-AUX1* and *CS-PIN1*. *Planta* 218: 15–26.

Kamiya, N., Ito, J., Morikami, A., Nagato, Y. and Matsuoka, M. (2003) The *SCARECROW* gene's role in asymmetric cell divisions in rice plants. *Plant J*. 36: 45–54.

Kato, T., Morita, M.T., Fukaki, H., Yamauchi, Y., Uehara, M., Niihama, M. and Tasaka, M.

(2002a) SGR2, a phospholipase-like protein, and ZIG/SGR4, a SNARE, are involved in the shoot gravitropism of *Arabidopsis*. *Plant Cell* 14: 33–46.

Kato, T., Morita, M.T. and Tasaka, M. (2002b) Role of endodermal cell vacuoles in shoot gravitropism. *J. Plant Growth Regul*. 21: 113–119.

Kimbrough, J.M., Salinas-Mondragon, R., Boss, W.F., Brown, C.S. and Sederoff, H.W. (2004) The fast and transient transcriptional network of gravity and mechanical stimulation in the *Arabidopsis* root apex. *Plant Physiol.* 136: 2790–2805.

Kiss, J.Z., Hertel, R. and Sack, F.D. (1989) Amyloplasts are necessary for full gravitropic sensitivity in roots of *Arabidopsis thaliana*. *Planta* 177: 198–206.

Kiss, J.Z. (2000) Mechanisms of the early phases of plant gravitropism. *Crit. Rev. Plant Sci.* 19: 551–573.

Kitazawa, D., Hatakeda, Y., Kamada, M., Fujii, N., Miyazawa, Y., Hoshino, A., Iida, S., Fukaki, H., Morita, M.T., Tasaka, M., Suge, H. and Takahashi, H. (2005) Shoot circumnutation and winding movements require gravisensing cells. *Proc. Natl. Acad. Sci. USA* 102: 18742–18747.

Kitazawa, D., Miyazawa, Y., Fujii, N., Nitasaka, E. and Takahashi, H. (2008) Characterization of a novel gravitropic mutant of morning glory, *weeping2*. *Adv. Space Res*. (in press, doi:10.1016/j.asr.2007.10.029)

Knight, T.A. (1806) On the direction of the radicle and germen during the vegetation of seeds. *Phil. Trans. R. Soc*. 99: 108–120.

Levesque, M.P., Vernoux, T., Busch, W., Cui, H., Wang, J.Y., Blilou, I., Hassan, H., Nakajima, K., Matsumoto, N., Lohmann, J.U., Scheres, B. and Benfey, P.N. (2006)

Whole-genome analysis of the SHORT-ROOT developmental pathway in *Arabidosis*. *PLoS Biol.* 4: 739–752.

Leyser, O. (2006) Dynamic integration of auxin transport and signalling. *Curr. Biol*. 16: R424–433.

Li, C.J., Guevara, E., Herrera, J. and Bangerth, F. (1995) Effect of apex excision and replacement by 1-naphthylacetic acid on cytokinin concentration and apical dominance in pea plants. *Physiol. Plant*. 94: 465–469.

Liscum, E. and Briggs, W.R. (1996). Mutations in *Arabidopsis* in potential transduction and response components of the phototropic signaling pathway. *Plant Physiol.* 112: 291–296.

Ljung, K., Bhalerao, R.P. and Sandberg, G. (2001) Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. *Plant J*. 28: 465–474.

Marchant, A., Kargul, J., May, S.T., Muller, P., Delbarre, A., Perrotrechenmann, C. and Bennett, M. J. (1999) AUX1 regulates root gravitropism in *Arabidopsis* by facilitating auxin uptake within root apical tissues. *EMBO J.* 18: 2066–2073.

Massa, G.D. and Gilroy, S. (2003) Touch modulates gravity sensing to regulate the growth of primary roots of *Arabidopsis thaliana. Plant J.* 33: 435–445.

Masson, P.H., Tasaka, M., Morita, M.T., Guan, C., Chen, R. and Boonsirichai, K. (2002) *Arabidopsis thaliana*: a model for the study of root and shoot gravitropism. In *The Arabidopsis Book*, Somerville, C.R. and Meyerowitz, E.M. (Eds.), American Society of Plant Biologists, Rockville, Maryland, doi/10.1199/tab.0043, http://www.aspb.org/publications/arabidopsis/.

Medford, J.I., Horgan, R., El-Sawi, Z. and Klee, H.J. (1989) Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. *Plant Cell* 1: 403–413.

Miyawaki, K., Matsumoto-Kitano, M. and Kakimoto, T. (2004) Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. *Plant J*. 37: 128–138.

Miyawaki, K., Tarkowski, P., Matsumoto-Kitano, M., Kato, T., Sato, S., Tarkowska, D., Tabata, S., Sandberg, G. and Kakimoto, T. (2006) Roles of *Arabidopsis* ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. *Proc. Natl. Acad. Sci. USA* 103: 16598–16603.

Mizuno, H., Kobayashi, A., Fujii, N., Yamashita, M. and Takahashi, H. (2002) Hydrotropic response and expression pattern of auxin-inducible gene, *CS-IAA1*, in the primary roots of clinorotated cucumber seedlings. *Plant Cell Physiol*. 43: 793–801.

Monschausen, G.B. and Sievers, A. (2002) Basipetal propagation of gravity-induced surface pH changes along primary roots of *Lepidium sativum* L. *Planta* 215: 980–988.

Morita, M.T., Kato, T., Nagafusa, K., Saito, C., Ueda, T., Nakano, A. and Tasaka, M. (2002) Involvement of the vacuoles of the endodermis in the early process of shoot gravitropism in *Arabidopsis*. *Plant Cell* 14: 47–56.

Morita, M.T. and Tasaka, M. (2004) Gravity sensing and signaling. *Curr. Opin. Plant Biol*. 7: 712–718.

Morita, Y., Saitoh, M., Hoshino, A., Nitasaka, E. and Iida, S. (2006a) Isolation of cDNAs for R2R3-MYB, bHLH and WDR transcriptional regulators and identification of *c* and *ca* mutations conferring white flowers in the Japanese morning glory. *Plant Cell Physiol*. 47:457–470.

Morita, M.T., Sakaguchi, K., Kiyose, S., Taira, K., Kato, T., Nakamura, M. and Tasaka, M. (2006b) A C2H2-type zinc finger protein, SGR5, is involved in early events of gravitropism in *Arabidopsis* inflorescence stems. *Plant J.* 47: 619–628.

Morita, M.T., Saito, C., Nakano, A. and Tasaka, M. (2007) *endodermal-amyloplast less 1* is a novel allele of *SHORT-ROOT*. *Adv. Space Res*. 39: 1127–1133.

Muday, G.K. (2001) Auxin and tropisms. *J. Plant Growth Regul*. 20: 226–243.

Nadella, V., Shipp, M.J., Muday, G.K. and Wyatt, S.E. (2006) Evidence for altered polar and lateral auxin transport in the *gravity persistent signal* (*gps*) mutants of *Arabidopsis*. *Plant Cell Environ*. 29: 682–690.

Nakagawa, Y., Katagiri, T., Shinozaki, K., Qi, Z., Tatsumi, H., Furuichi, T., Kishigami, A., Sokabe, M., Kojima, I., Sato, S., Kato, T., Tabata, S., Iida, K., Terashima, A., Nakano, M., Ikeda, M., Yamanaka, T. and Iida, H. (2007) *Arabidopsis* plasma membrane protein crucial for Ca^{2+} influx and touch sensing in roots. *Proc. Natl. Acad. Sci. USA 27*: 3639–3644.

Nakajima, K., Sena, G., Nawy, T. and Benfey, P.N. (2001) Intercellular movement of the putative transcription factor SHR in root patterning. *Nature* 413: 307–311.

Niinuma, K., Someya, N., Kimura, M., Yamaguchi, I. and Hamamoto, H. (2005) Circadian rhythm of circumnutation in inflorescence stems of *Arabidopsis*. *Plant Cell Physiol*.46: 1423–1427.

Niklas, K.J. (1992) Gravity-induced effects on material properties and size of leaves on horizontal shoots of *Acer saccharum* (Aceraceae). *Amer. J. Bot*. 79:820–827.

Noh, B., Bandyopadhyay, A., Peer, W.A., Spalding, E.P. and Murphy, A.S. (2003) Enhanced gravi- and phototropism in plant *mdr* mutants mislocalizing the auxin efflux protein PIN1. *Nature* 424: 999–1002.

Nordström, A., Tarkowski, P., Tarkowska, D., Norbaek, R., Åstot, C., Dolezal, K. and Sandberg, G. (2004) Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: a factor of potential importance for auxin-cytokinin-regulated development. *Proc. Natl. Acad. Sci. USA* 101: 8039–8044.

Okada, K. and Shimura, Y. (1992) Mutational analysis of root gravitropism and phototropism of *Arabidopsis thaliana* seedlings. *Aust. J. Plant Physiol*. 19: 439–448.

Ongaro, V. and Leyser, O. (2007) Hormonal control of shoot branching. *J. Exp. Bot*. (doi: doi:10.1093/jxb/erm134)

Ottenschlager, I., Wolff, P., Wolverton, C., Bhalero, R.P., Sandberg, G., Ishikawa, H., Evans, M.L. and Palme, K. (2003) Gravity-regulated differential auxin transport from columella to lateral root cap cells. *Proc. Natl. Acad. Sci. USA* 100: 2987–2991.

Paciorek, T., Zazímalová, E., Ruthardt, N., Petrásek, J., Stierhof, Y.D., Kleine-Vehn, J., Morris, D.A., Emans, N., Jürgens, G., Geldner, N. and Friml, J. (2005) Auxin inhibits endocytosis and promotes its own efflux from cells. *Nature* 30:1251–1256.

Palme, K., Dovzhenko, A. and Ditengou, F.A. (2006) Auxin transport and gravitational research: perspectives. *Protoplasma* 229: 175–181.

Perera, I.Y., Hung, C.Y., Brady, S., Muday, G.K. and Boss, W.F. (2006) A universal role for inositol 1,4,5-trisphosphate-mediated signaling in plant gravitropism. *Plant Physiol*. 140: 746–760.

Pillay, I. and Railton, I.D. (1983) Complete release of axillary buds from apical dominance in intact, light-grown seedlings of *Pisum sativum* L. following a single application of cytokinin. *Plant Physiol*. 71: 972–974.

Ripetskyj, R.T., Kit, N.A. and Chaban, C.I. (1998) Gravity effects on the growth and development of moss secondary protonemata. *Adv. Space Res*. 21: 1135–1139.

Plieth, C. and Trewavas, A.J. (2002) Reorientation of seedlings in the earth's gravitational field induces cytosolic calcium transients. *Plant Physiol.* 129: 786–796.

Prasad, T.K. and Cline, M.G. (1985) Shoot inversion-induced ethylene in *Pharbitis nil* induces the release of apical dominance by restricting shoot elongation. *Plant Sci.* 38:163–172.

Prasad, T.K. and Cline, M.G. (1987) The role of gravity in apical dominance. *Plant Physiol*. 83: 505–509.

Prasad, T.K., Li, X., Abdel-Rahman, A.M., Hosokawa, Z., Cloud, N.P., LaMotte, C.E. and Cline, M.G. (1993) Does auxin play a role in the release of apical dominance by

shoot inversion in *Ipomoea nil*? *Ann. Bot*. 71: 223–229.

Prasad, T.K. and Cline, M.G. (1987) The role of gravity in apical dominance. *Plant Physiol*. 83: 505–509.

Pysh, L.D., Wysocka-Diller, J., Camilleri, C., Bouchez, D. and Benfey, P.N. (1999) The GRAS gene family in *Arabidopsis*: sequence characterization and basic expression analysis of the *SCARECROW-LIKE* genes. *Plant J.* 18: 111–119.

Romano, C.P., Cooper, M.L. and Klee, H.J. (1993) Uncoupling auxin and ethylene effects in transgenic tobacco and *Arabidopsis* plants. *Plant Cell* 5: 181–189.

Sabatini, S., Heidstra, R., Wildwater, M. and Scheres, B. (2003) SCARECROW is involved in positioning the stem cell niche in the *Arabidopsis* root meristem. *Genes Dev*. 17: 354–358.

Sack, F.D. (1997) Plastids and gravitropic sensing. *Planta* 203: S63–S68.

Saito, Y., Yamasaki, S., Fujii, N., Hagen, G., Guilfoyle, T. and Takahashi, H. (2004) Isolation of cucumber *CsARF* cDNAs and expression of the corresponding mRNAs during gravity-regulated morphogenesis of cucumber seedlings. *J. Exp. Bot*. 55: 1315-1323.

Saito, C., Morita, M.T., Kato, T. and Tasaka, M. (2005) Amyloplasts and vacuolar membrane dynamics in the living graviperceptive cell of the *Arabidopsis* inflorescence stem. *Plant Cell* 17: 548–558.

Scher, J.L., Holbrook, N.M. and Silk, W.K. (2001) Temporal and spatial patterns of twining force and lignification in stems of *Ipomoea purpurea*. *Planta* 213: 192–198.

Schuster, J. and Engelmann, W. (1997) Circumnutations of *Arabidopsis thaliana* seedlings. *Biol. Rhythm Res*. 28: 422–440.

Scott, A.C. and Allen, N.S. (1999) Changes in cytosolic pH within *Arabidopsis* root columella cells play a key role in the early signaling pathway for root gravitropism. *Plant Physiol.* 121: 1291–1298.

Shimizu-Sato, S. and Mori, H. (2001) Control of outgrowth and dormancy in axillary buds. *Plant Physiol*. 127: 1405–1413.

Shimizu, M., Suzuki, K., Miyazawa, Y., Fujii, N. and Takahashi, H. (2006) Differential accumulation of the mRNA of the auxin-repressed gene *CsGRP1* and the auxin-induced peg formation during gravimorphogenesis of cucumber seedlings. *Planta* 225: 13–22.

Shimizu, M., Miyazawa, Y., Fujii, N. and Takahashi, H. (2007) *p*-Chlorophenoxyisobutyric acid impairs auxin response for gravity-regulated peg formation in cucumber (*Cucumis sativus*) seedlings. *J. Plant Res.* (doi: 10.1007/s10265-007-0121-0)

Shin, H., Shin, H.S., Guo, Z., Blancaflor, E.B., Masson, P.H. and Chen, R. (2005) Complex regulation of *Arabidopsis* AGR1/PIN2-mediated root gravitropic response and basipetal auxin transport by cantharidin-sensitive protein phosphatases. *Plant J.* 42:188–200.

Silady, R.A., Kato, T., Lukowitz, W., Sieber, P., Tasaka, M. and Somerville, C.R. (2004) The *gravitropism defective 2* mutants of *Arabidopsis* are deficient in a protein

implicated in endocytosis in *Caenorhabditis elegans*. *Plant Physiol*. 136: 3095–3103.

Skoog, F. and Thimann, K.V. (1934) Further experiments on the inhibition of the development of lateral buds by growth hormone. *Proc. Natl. Acad. Sci. USA* 20: 480–485.

Snowden, K.C. and Napoli, C.A. (2003) A quantitative study of lateral branching in petunia. *Funct. Plant Biol*. 30: 987–994.

Soga, K., Wakabayashi, K., Hoson, T. and Kamisaka, S. (2001) Gravitational force regulates elongation growth of *Arabidopsis* hypocotyls by modifying xyloglucan metabolism. *Adv. Space Res*. 27: 1011–1016.

Soga, K., Wakabayashi, K., Kamisaka, S. and Hoson, T. (2002) Stimulation of elongation growth and xyloglucan breakdown in *Arabidopsis* hypocotyls under microgravity conditions in space. *Planta* 215: 1040–1046.

Soga, K., Wakabayashi, K., Kamisaka, S. and Hoson, T. (2006) Hypergravity induces reorientation of cortical microtubules and modifies growth anisotropy in azuki bean epicotyls. *Planta* 224: 1485–1494.

Someya, N., Niinuma, K., Kimura, M., Yamaguchi, I. and Hamamoto, H. (2006) Circumnutation of *Arabidopsis thaliana* inflorescence stems. *Biol. Plant*. 50: 287–290. (brief communication)

Stirnberg, P., Chatfield, S.P. and Leyser, H.M. (1999) AXR1 acts after lateral bud formation to inhibit lateral bud growth in *Arabidopsis*. *Plant Physiol.* 121: 839–847.

Swarup, R., Kargul, J., Marchant, A., Zadik, D., Rahman, A., Mills, R., Yemm, A., May, S., Williams, L., Millner, P., Tsurumi, S., Moore, I., Napier, R., Kerr, I.D. and Bennet, M.J. (2004) Sturcture-function analysis of the presumptive *Arabidopsis* auxin permease AUX1. *Plant Cell* 16: 3069–3083.

Takahashi, H. and Scott, T.K. (1994) Gravity-regulated formation of the peg in developing cucumber seedlings. *Planta* 193: 580–584.

Takahashi, H. (1997) Gravimorphogenesis: gravity-regulated formation of the peg in cucumber seedlings. *Planta* 203: S164–S169.

Takahashi, H., Mizuno, H., Kamada, M., Fujii, N., Higashitani, A., Kamigaichi, S., Aizawa, S., Mukai, C., Shimazu, T., Fukui, K. and Yamashita, M. (1999) A spaceflight experiment for the study of gravimorphogenesis and hydrotropism in cucumber seedlings. *J. Plant Res*. 112: 497–505.

Takahashi, H., Fujii, N., Kamada, M., Higashitani, A., Yamazaki, Y., Kobayashi, A., Takano, M., Yamasaki, S., Sakata, T., Mizuno, H., Kaneko, Y., Murata, T., Kamigaichi, S., Aizawa, S., Yoshizaki, I., Shimazu, T. and Fukui, K. (2000) Gravimorphogenesis of Cucurbitaceae plants: development of peg cells and graviperception mechanism in cucumber seedlings. *Biol. Sci. Space* 14: 64–74.

Tanaka, M., Takei, K., Kojima, M., Sakakibara, H. and Mori, H. (2006) Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. *Plant J*. 45: 1028–1036.

Tepper, H.B. and Yang, R.L. (1996) Influence of the shoot tip and leaves on circumnutation in green pea seedlings. *Bot. Acta*. 109: 502–505.

Tian, C., Wan, P., Sun, S., Li, J. and Chen, M. (2004) Genome-wide analysis of the GRAS gene family in rice and *Arabidopsis*. *Plant Mol. Biol.* 54: 519–532.

Tiwari, S.B., Hagen, G. and Guilfoyle, T.J. (2004) Aux/IAA proteins contain a potent transcriptional repression domain. *Plant Cell* 16: 533–543.

Wagner, T.A. and Sack, F.D. (1998) Gravitropism and gravimorphism during regeneration from protoplasts of the moss *Ceratodon purpureus* (Hedw.) Brid. *Planta* 205: 352–358.

Wareing, P.F. and Nasr, T.A.A. (1958) Gravimorphism in trees: effects of gravity on growth, apical dominance and flowering in fruit trees. *Nature* 182: 379–380.

Weiler, E.W. (1980) Radioimmunoassays for *trans*-zeatin and related cytokinins. *Planta* 149: 155–162.

Weiler, E.W. (1984) Immunoassay of plant growth regulators. *Ann. Rev. Plant Physiol*. 35: 85–95.

Wright, M., Mousdale, D.M. and Osborne, D.J. (1978) Evidence for a gravity-regulated level of endogenous auxin controlling cell elongation and ethylene production during geotropic bending in grass nodes. *Biochem. Physiol. Pflanzen.* 172: 581–596.

Wright, M. (1981) Reversal of the polarity of IAA transport in the leaf sheath base of *Echinochloa colonum*. *J. Exp. Bot*. 32: 159–169.

Wysocka-Diller, J.W., Helariutta, Y., Fukaki, H., Malamy, J.E. and Benfey, P.N. (2000) Molecular analysis of SCARECROW function reveals a radial patterning mechanism common to root and shoot. *Development* 127: 595–603.

Yamamoto, K. and Kiss, J.Z. (2002) Disruption of the actin cytoskeleton results in the promotion of gravitropism in inflorescence stems and hypocotyls of *Arabidopsis. Plant Physiol.* 128: 669–681.

Yano, D., Sato, M., Saito, C., Sato, M.H., Morita, M.T. and Tasaka, M. (2003) A SNARE complex containing SGR3/AtVAM3 and ZIG/VTI11 in gravity-sensing cells is important for *Arabidopsis* shoot gravitropism. *Proc. Natl. Acad. Sci. USA* 100: 8589–8594.

Yoder, T.L., Zheng, H.Q., Todd, P. and Staehelin, L.A. (2001) Amyloplast sedimentation dynamics in maize columella cells support a new model for the gravity-sensing apparatus of roots. *Plant Physiol.* 125: 1045–1060.

Yoshihara, T. and Iino, M. (2005) Circumnutation of rice coleoptiles: its occurrence, regulation by phytochrome, and relationship with gravitropism. *Plant Cell Environ*. 28: 134–146.

Yoshihara, T. and Iino, M. (2006) Circumnutation of rice coleoptiles: its relationships with gravitropism and absence in *lazy* mutants. *Plant Cell Environ*. 29: 778–792.

Young, L.S., Harrison, B.R., Narayana-Murthy U.M., Moffatt, B.A., Gilroy, S. and Masson, P.H. (2006) Adenosine kinase modulates root gravitropism and cap morphogenesis in *Arabidopsis*. *Plant Physiol*. 142: 564–573.

Zubko, E., Adams, C.J., Macháèková, I., Malbeck, J., Scollan, C. and Meyer, P. (2002) Activation tagging identifies a gene from *Petunia hybrida* responsible for the production of active cytokinins in plants. *Plant J*. 29: 797–808.

Daisuke Kitazawa

January 2008

Laboratory of Space and Adaptation Biology, Department of Environmental Life Sciences, Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan

Tel: 022-217-5726

Fax: 022-723-8218